Spatiotemporal replication dynamics of *Salmonella* during systemic disease

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I certify that this report is my own work and that all else is appropriately referenced

ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) causes a systemic disease in susceptible mouse strains that is widely used as a model of human typhoid fever. I developed a reporter system based on fluorescence dilution that measures bacterial replication directly at both the population and single cell level. To understand how *S.* Typhimurium colonises host tissues during disease, I applied fluorescence dilution to study *S.* Typhimurium replication during acute murine typhoid, following oral inoculation. Bacteria that had not replicated were found in the Peyer's patches (PP), mesenteric lymph nodes (MLN) and spleen. Hence, replication is not a prerequisite for *S.* Typhimurium to traverse the intestinal wall and reach deeper tissues. Furthermore, non-replicating bacteria were found to persist for long periods of time within the intestine and, to a smaller extent, in the spleen, suggesting that these bacteria may represent dormant reservoirs that cause chronic infections. Bacteria replicated rapidly upon invasion of the PP, while the MLN represented a more restrictive niche for bacterial replication. In addition, the spleen was seeded preferentially by bacteria that had already replicated elsewhere. Further analysis of bacterial replication in the spleen provided insights into the contribution of the *Salmonella*-encoded pathogenicity island-2 type III secretion system to replication in this organ. Experiments also showed that *S.* Typhimurium preferentially colonised and replicated within macrophages within the spleen. Therefore, the use of fluorescence dilution has provided detailed insights into the relationship between the replication of *S.* Typhimurium and its colonisation of host tissues, and has revealed the existence and localisation of non-replicating bacteria that could persist during chronic infections.

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ABBREVIATIONS

AI	autoinducer
Amp^R	Ampicillin resistance
ATR	acid tolerance response
bm	bone marrow-derived
bp	base pair
CD	cluster of differentiation factor
$CD8^+$ T-lymphocyte	CTL
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming units
CI	competitive index
CR	complement receptor
CSA-1	*Salmonella* common structural antigen-1
CtsD	Cathepsin D
DC	dendritic cell
DIC	differential interference contrast
DiGc	DsRed-inducible GFP-constitutive
DiGi	DsRed-inducible GFP-inducible
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DsRed	*Discosoma* Red fluorescent protein
EDTA	ethylenediaminetetraacetic acid
F-actin	filamentous actin
FACS	fluorescence-activated cell sorting
FAE	follicle-associated epithelium
FCcGi	FPV25 backbone mCherry-constitutive GFP-inducible
FCiGc	FPV25 backbone mCherry-inducible GFP-constitutive
FcR	fragment, crystallisable region receptors for immunoglobulin
FCS	heat inactivated fetal calf serum
FSC	forward scatter
GAP	GTPase activating protein
GEF	guanidine nucleotide exchange factor
GFP	Green fluorescent protein
GTPase	guanine nucleotide triphosphatases
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
HRP	Horse radish peroxidase
HS	horse serum
i.g.	intragastric
i.p.	intraperitoneal
i.v.	intravenous
IFNγ	interferon-gamma
IgG2b	immunoglobulin class G2b
IL	interleukin
iNOS	inducible nitric oxide synthase

IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
LAMP	lysosome-associated membrane protein
LB	Luria-Bertani
LD	lethal dose
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDR	multi-drug resistant
MFI	geometric mean of fluorescence intensity
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MM	MgMES minimal medium
moi	multiplicity of infection
ND	not done
NF-κB	nuclear factor- kappa beta
NR	non-replicating
NTS	nontyphoidal salmonellosis
OD	optical density
p	probability
p.i.	post-inoculation
PAGE	polyacrylamide gel electrophoresis
pags	PhoP-activated genes
PAI	pathogenicity island
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PhoPQ	PhoP/PhoQ
Phox	phagocyte oxidase
PMN	polymorphonuclear leukocyte
PP	Peyer's patches
prgs	PhoP-repressed genes
PRR	pattern-recognition receptor
pSLT	*Salmonella* virulence plasmid
RNS	reactive nitrogen species
rop	*repressor of primer*
ROS	reactive oxygen species
RT	room temperature
rγ	interleukin receptor gamma subunit
SCV	*Salmonella*-containing vacuole
SDS	sodium dodecyl sulphate
SEM	standard error from the mean
SI	small intestinal lumen
Sif	*Salmonella*-induced filament
Slc11a1	solute carrier 11a1
SOB	super optimal broth
SOC	super optimal broth with catabolite repression

SPI	*Salmonella*-pathogenicity island
spv	*Salmonella* plasmid virulence
SSC	side scatter
t_{0h}	time = 0 h
T3SS	type three secretion system
TAE	Tris-acetate-EDTA
Th	T helper type
TLR	toll-like receptor
TNFα	tumour-necrosis factor-alpha
tRNA	transfer ribonucleic acid gene
Vi CPS	Vi capsular polysaccharide
WITS	wild-type isogenic tagged strains
wt	wild-type

1. INTRODUCTION

1.1. Classification and evolution of *Salmonella*

Salmonella is a genus of Gram-negative, facultative anaerobic bacilli that belongs to the family of Enterobacteriaceae. These bacilli were first isolated by Gaffkey in 1884 and later named after Daniel Salmon. The nomenclature used to define this genus was previously very complex, however multilocus enzyme electrophoresis and genomic deoxyribonucleic acid (DNA)-DNA hybridisation have since been used to provide a more accurate and universally acceptable classification. *Salmonella* is now known to consist of three species: *Salmonella bongori* (Reeves *et al.*, 1989, Boyd *et al.*, 1996), *Salmonella enterica* (Crosa *et al.*, 1973, Le Minor and Popoff, 1987) and *Salmonella subterranean* (Shelobolina *et al.*, 2004) (Fig. 1.1).

Analysing the phylogenetic distribution of *Salmonella* genes has revealed much information about the evolution of this genus and the development of *S. enterica* pathogenicity. Approximately 120-160 million years ago, *Salmonella* diverged from the genus *Escherichia* and soon after, acquired products that enabled enteropathogenicity. These include factors that enable attachment to the intestinal epithelium, such as: type I fimbriae and long polar fimbriae, encoded within the *fim* and *lpf* are operons, respectively, and factors that mediate invasion of the intestinal epithelium (Mills *et al.*, 1995, Li *et al.*, 1995, Baumler *et al.*, 1997a). The latter consists of genes contained within *Salmonella*-pathogenicity island-1 (SPI-1), which encodes the machinery and secreted proteins of a type three secretion system (T3SS) (Galan and Curtiss, 1989). As a result, *Salmonella* was able to access a more favourable niche within the intestinal epithelium and subepithelial layer, where there was presumably less competition for nutrients and colonisation sites compared to the commensal-rich environment of the gut lumen.

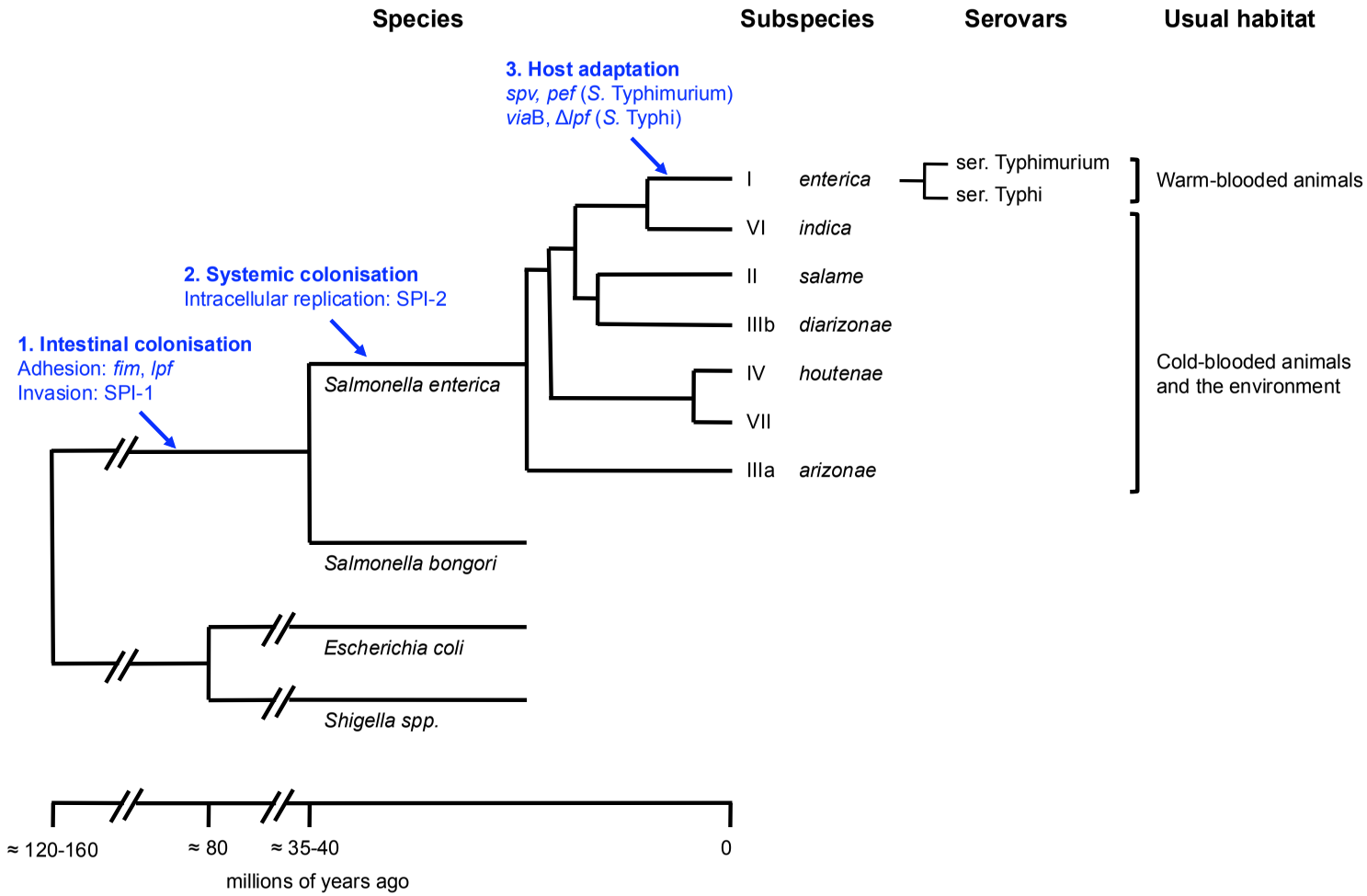


Figure 1.1. Phylogenetic relationships between *Salmonella* subspecies.
Adapted from Bäumler (1997) and Cotter and DiRita (2000).

The next evolutionary transition resulted in the divergence of *Salmonella enterica* from *Salmonella bongori*. This correlates with the acquisition of a second T3SS, encoded by genes within SPI-2, which promotes intracellular replication (Shea *et al.*, 1996, Ochman *et al.*, 1996, Hensel *et al.*, 1997). Using this virulence system, *Salmonella enterica* was able to better colonise host tissues and disseminate from the gastrointestinal tract to systemic sites, by exploiting a relatively protected niche within host cells.

Salmonella enterica underwent further diversification, through multifactorial acquisition and loss of genetic elements, which gave rise to an expansion of the host range. As a result, this species has been classified into six subspecies: I (*enterica*), II (*salamae*), IIIa (*arizonnae*), IIIb (*diarizonae*), IV (*houtenae*), VI (*indica*) and VII (not yet named) (Boyd *et al.*, 1996, Crosa *et al.*, 1973). Before being recognised as a separate species, *Salmonella bongori* was classified as subspecies V. All subspecies, apart from group I, typically inhabit cold-blooded vertebrates and the environment. However, group I or *Salmonella enterica* subspecies *enterica* (*S. enterica*) gained the ability to colonise warm-blooded vertebrates. *S. enterica* has been further divided into over 2400 serovars that can be serologically identified using the Kaufmann and White classification scheme. This involves grouping isolates according to their expression of three highly variable surface antigens: lipopolysaccharide (LPS) 'O' antigen, flagellar 'H' antigen and virulence 'Vi' polysaccharide capsular antigen.

Serovars of *S. enterica* display varying degrees of host specificity, resulting in a wide spectrum of gastrointestinal to systemic diseases (Table 1.1). Clinical manifestation of disease depends on both the serovar and the host. *S. enterica* Typhi (*S.* Typhi) and *S.* Paratyphi A, B and C are host-restricted and only infect humans and higher primates, where they cause an acute systemic illness known as typhoid and paratyphoid fever, respectively. Host-adapted serovars include *S.* Dublin, *S.* Choleraesuis and *S.* Gallinarum. These can infect several hosts, however they cause the majority of *Salmonella*-associated disease in cattle (*S.* Dublin), pigs (*S.* Choleraesuis) and chickens (*S.* Gallinarum), suggesting a greater adaptation to these hosts (Kingsley and

Bäumler, 2000). Other serovars are generalists, such as *S.* Typhimurium, which can infect a broad host range including humans, cattle, pigs, poultry, horses and rodents.

S. enterica serovar	Human disease	Disease in other hosts
Typhi	Typhoid	Higher primates (typhoid)
Paratyphi	Paratyphoid	Higher primates (paratyphoid)
Typhimurium	Colitis	Mice (murine typhoid) Wide range e.g. Cattle, Pigs (enteritis)
Enteriditis	Colitis	Wide range e.g. Cattle, Pigs (enteritis)
Dublin	Invasive bacteraemia	Cattle (bovine typhoid)
Choleraesuis	Invasive bacteraemia	Pigs (swine typhoid)
Gallinarum	(Rare)	Chickens (fowl typhoid)

Table 1.1. Spectrum of diseases caused by serovars of *S. enterica.*

1.2. Human diseases caused by *S. enterica*

In humans, S. *enterica* is responsible for the vast majority (>99%) of *Salmonella*-associated infections (Baumler, 1997), with only 12 serovars causing approximately 70% of reported cases (Anjum *et al.*, 2005). The major clinical syndromes in immunocompetent humans that result from infection with *S. enterica* are enteric (typhoid and paratyphoid) fever and colitis (Table 1.2). However, in certain risk groups, including young, elderly and immunocompromised individuals, bacteriaemia and other focal infections also occur.

	Diarrhoeal disease	Typhoid fever
Incubation period	Short (12-72 h)	Long (8-14 days)
Symptom duration	Short (3-7 days)	Long (2-3 weeks untreated)
Intestinal pathology	Colitis PMN infiltrate	Enteritis (mild) Mononuclear infiltrate
Extraintestinal pathology	MLN	MLN, Liver, Spleen, Bone marrow
Complications	Bacteraemia	Haemorrhage (10-20%) Intestinal perforation (1-3%)
Outcome	Typically self-limiting	Mortality (<1% if treated) Chronic carriage (1-5%) Relapse (5-10%)

Table 1.2. Common features of NTS and typhoid fever in humans.
PMN (polymorphonuclear leukocyte); MLN (mesenteric lymph nodes)

1.2.1. Nontyphoidal salmonellosis

1.2.1.1. Diarrhoeal disease (Colitis)

S. enterica-induced diarrhoeal disease or colitis in humans represents a significant disease burden and gives rise to approximately 3% (93.8 million cases) of diarrhoeal diseases worldwide each year, with 155 000 fatalities

(Majowicz *et al.*, 2010). Many nontyphoidal *S. enterica* serovars can cause human colitis. The most prevalent isolates in the US and parts of Europe are *S.* Enteriditis and *S.* Typhimurium (Olsen *et al.*, 2001, Cogan and Humphrey, 2003). These bacteria can colonise the intestines of most farm animals and therefore, can be acquired from multiple animal reservoirs. Transmission is usually by consumption of food, such as eggs, meat and dairy products from infected animals or via food or water that has become contaminated with animal waste.

In healthy adults, nontyphoidal salmonellosis (NTS) is usually self-limiting (Table 1.2). Symptoms typically manifest after a short incubation period of 12 to 72 h and these consist of nausea, vomiting, diarrhoea and abdominal cramps (Pegues and Miller, 2010). Acute diffuse colitis with crypt abscess formation and occasionally distal ileal involvement is typically observed by post-mortem or biopsy (Day *et al.*, 1978, McGovern and Slavutin, 1979, Boyd, 1985). This acute inflammation is also characterised by mucosal oedema and a large polymorphonuclear leukocyte (PMN) infiltrate. The majority of cases resolve without treatment within 3 to 7 days, however some patients can become chronic fecal carriers (Buchwald and Blaser, 1984).

1.2.1.2. Extraintestinal infections

Infants, elderly and immunocompromised individuals with NTS are often unable to contain the infection to the gastrointestinal tract and in these cases a potentially life-threatening invasive disease can occur. This syndrome is particularly prevalent in human immunodeficiency virus (HIV)-infected individuals in sub-Saharan Africa, where mortality rates can reach approximately 50% and many patients experience recurrent infections (Gordon *et al.*, 2002). Invasive NTS bacteraemia is frequently associated with a non-specific fever and hepato- and splenomegaly, in the absence of gastrointestinal symptoms (Gordon, 2008). Approximately 5-10% of invasive NTS infections develop metastatic focal infections (Mandal and Brennand, 1988), with increased incidence rates observed in immunosuppressed

individuals. Vascular infections are the most common, however disease can manifest in almost all organ systems.

1.2.2. Typhoid fever

S. Typhi and *S.* Paratyphi are the causative agents of the enteric fevers known as typhoid and paratyphoid fever, respectively. Typhoid is a more severe disease then paratyphoid and is responsible for the vast majority (approximately 90%) of enteric fever (Parry, 2006). In 2000 alone, typhoid is estimated to have caused approximately 21 million cases and 200 000 deaths worldwide (Crump *et al.*, 2004). However, this is likely to represent an underestimation of disease rates, as endemic areas often lack the facilities to provide a definitive diagnosis. Infections result from ingestion of food or water contaminated with human waste. Therefore, typhoid is more predominant in developing countries, which lack the infrastructure to provide adequate sanitation and access to clean drinking water. The greatest burden of typhoid is within regions of south-central and southeastern Asia and southern Africa (Crump *et al.*, 2004).

The infectious dose of *S.* Typhi in healthy human volunteers is between 10^3 to 10^9 organisms (Hornick *et al.*, 1970). Gastric acid provides the initial barrier to infection and an increased susceptibility to typhoid commonly occurs in patients with achlorhydria, due to ageing, previous gastrectomy, treatments affecting gastric acid production or infection with *Helicobacter pylori* (*H. pylori*) (Bhan *et al.*, 2002). A broad range of symptoms has been reported in typhoid patients. These differ in severity and normally develop following a longer incubation period of 8 to 14 days, compared to NTS infections (Table 1.2). Early symptoms include diarrhoea, common in young children and adults with HIV infection, and fever (Parry, 2006). Constipation is also common in about one third of patients (Pegues and Miller, 2010). Other common symptoms include abdominal pain, malaise, flu-like symptoms and headache. Intestinal biopsies reveal enteritis and mucosal thickening characterised by mononuclear-rich infiltrates (Sprinz *et al.*, 1966, Kraus *et al.*, 1999). Most patients develop a low-grade bacteraemia, thought to facilitate the

dissemination of *S.* Typhi to systemic sites after invasion of the Peyer's patches (PP) (Hornick et al., 1970). Enlargement of the mesenteric lymph nodes (MLN), liver and spleen is often observed and is associated with the formation of granulomatous lesions. Bacteria are also found in the bone marrow and gallbladder. Importantly, re-seeding of *S.* Typhi from an infected gallbladder back into the intestine can lead to re-invasion of the PP (Bos and Willemsen, 2002). This can cause serious complications, namely: capillary thrombosis of the terminal ileal PP, resulting in haemorrhage (10-20%), necrosis, ulceration and eventually intestinal perforation (1-3%) (Pegues and Miller, 2010). Untreated typhoid usually resolves in approximately 3 weeks, however relapse occurs in 5-10% of cases (Parry, 2006). With good management, that is: eradication of the infection with antibiotics, rehydration, restoration of nutrition and treatment of complications, typhoid fatality rates can be as low as 1% (Pegues and Miller, 2010). However, antibiotic treatment is becoming gradually more challenging, with the increasing incidence of drug and multi-drug resistant (MDR) serovars (Crump and Mintz, 2010).

Following acute typhoid fever, approximately 1-5% of patients develop chronic asymptomatic infection characterised by persistent shedding of S. Typhi in urine or stools (Bhan *et al.*, 2005). Approximately 25% of chronic carriers arise from subclinical infections of S. Typhi (Mohan *et al.*, 2006). Persistent reservoirs of bacteria are thought to be located within the liver (Nath *et al.*, 2010) and gallbladder (Sinnott and Teall, 1987). Adequate surveillance for such individuals is important, as they can contribute significantly to the spread of infection. In addition, long-term carriage of *S. enterica* has been suggested to increase the risk of gallbladder, pancreas and large bowel carcinoma (Caygill *et al.*, 1994).

1.2.3. Disease prevention

Given that *S.* Typhi can only survive within human hosts and is transmitted between hosts via contaminated food and water, it would be possible to eradicate typhoid. Indeed, this has almost been achieved in developed countries, where the majority of typhoid cases only occur in returning

travellers (Clark *et al.*, 2010). However, the worldwide eradication of typhoid is not currently feasible, as this disease is endemic in many countries that still lack the means to provide safe drinking water and basic sanitation. Therefore, one measure to control the incidence of typhoid fever has been the development of vaccines against *S.* Typhi. Two vaccines are currently licensed against typhoid fever: Ty21a, a live attenuated oral vaccine, which must be adminstered as 3 to 4 doses and a parenteral Vi capsular polysaccharide (Vi CPS), consisting of a purified polysaccharide from the bacterial capsule. Neither vaccine exhibits 100% efficacy (Fraser *et al.*, 2007), nor can they be given to very young children, an age-group with high incidence rates of typhoid fever in endemic countries (Sinha *et al.*, 1999). A Vi polysaccharide-protein conjugate, a conjugate of Vi CPS with non-toxic recombinant *Pseudomonas aeruginosa* endotoxin, represents a promising new vaccine with improved efficacy in children (Lin *et al.*, 2001). Other candidates in clinical trials include attenuated *S.* Typhi strains with combinations of several mutations or deletions in genes required for biosynthetic pathways and/or virulence systems, such as the M01ZH09 *S.* Typhi vaccine strain that harbours an *aroC* and *ssaV* deletion, which are required for aromatic amino acid biosynthesis and SPI-2 T3SS-associated effector secretion, respectively (Kirkpatrick *et al.*, 2006, Hien *et al.*, 2010), with the aim of creating a vaccine that is effective after a single oral dose. No vaccine is currently available against *S.* Paratyphi or nontyphoidal serovars.

In summary, *S. enterica* places a major burden on public health services and economy worldwide. Case-fatality rates can be high and disease management can be difficult, particularly with regard to the global increase of MDR isolates and the persistence of chronic carriers. Furthermore, an ideal vaccine is yet to be found. Together, this highlights the need for a better understanding of *S. enterica* pathogenesis, so that more efficient control and treatment regimens can be developed.

1.3. Animal models of human *S. enterica* infections

Use of appropriate animal models represents a fundamental part of *S. enterica* research. Although a wealth of knowledge can be gained from tissue culture models, the relevance of these findings must be tested in more physiological contexts. Furthermore, certain aspects of pathogenesis, such as systemic dissemination and tissue colonisation, can only be properly studied within the living host.

1.3.1. Models of *S. enterica*-induced colitis

Most studies of NTS in man have focused on experimental infection of calves with *S.* Typhimurium, as this produces a pathology in the intestine that is similar to human colitis (Santos *et al.*, 2001). In particular, the bovine ligated intestinal loop model has provided insights into the early dynamics of intestinal pathology and the contribution of *S. enterica* virulence factors to the development of intestinal disease, for example the requirement of the SPI-1 T3SS for invasion and fluid secretion (Layton and Galyov, 2007). However, to overcome the out-bred nature, high cost and lack of tools that limit analyses using bovine models, a murine model has been developed (Barthel *et al.*, 2003). Although *S.* Typhimurium produces a systemic disease with minimal intestinal involvement in murine hosts, pre-treatment with the antibiotic streptomycin eliminates the gut microflora and renders the intestine susceptible to colonisation. These mice subsequently develop acute diffuse inflammation of the caecum and colon, accompanied by a PMN-rich infiltrate. In susceptible mouse strains, this intestinal pathology occurs in parallel with systemic typhoid, which limits studies to early time-points. However, this has been somewhat overcome by using genetically-resistant mice (Stecher *et al.*, 2006, Woo *et al.*, 2008). Streptomycin pre-treated mice have since been used to study the role of *S. enterica* effectors and host immune responses in colitis pathogenesis (Hapfelmeier *et al.*, 2004, Coburn *et al.*, 2005, Hapfelmeier *et al.*, 2008, Valdez *et al.*, 2009).

1.3.2. Murine model of typhoid fever

The pathogenesis of typhoid fever has been difficult to study, owing to the host restriction of *S.* Typhi. Some of the first typhoid fever studies were conducted in chimpanzees, which are susceptible to infection with *S.* Typhi (Edsall *et al.*, 1960). Although these primates display a milder form of typhoid, the clinical course of infection and pathological tissue changes are very typical of the human disease (Edsall *et al.*, 1960, Gaines *et al.*, 1968).

However, over the last 20 years, research has largely focused on the closely related strain *S.* Typhimurium, in a murine model of systemic salmonellosis. *S.* Typhimurium is a natural pathogen of mice and certain genetically susceptible, inbred mouse strains develop a systemic pathology that closely resembles human infection with *S.* Typhi. Using mice is more cost efficient than large animal models. In addition, the genetics of mouse strains is well developed. Therefore, murine infection with *S.* Typhimurium has been widely adopted as a surrogate pathogen and host model to study the bacterial virulence and host resistance mechanisms that occur during human typhoid fever.

Experimental infection of BALB/c and C57BL/6 natural resistance-associated macrophage protein-1 (now known as solute carrier 11a1 [Slc11a1])-deficient mouse strains are used to model acute typhoid fever. These mice carry a mutation that inactivates Slc11a1 (Lissner *et al.*, 1983, Vidal *et al.*, 1993), rendering them highly susceptible to *S.* Typhimurium infection. Acute *S.* Typhimurium infections in Slc11a1-susceptible mice have been well characterised. Oral infection of these mice with 10^4 to 10^5 colony forming units (CFU) of *S.* Typhimurium results in a fatal systemic disease (Scherer and Miller, 2001). Considerably less bacteria (<10 CFU) are required to cause death in 50% of infected mice (lethal dose, LD_{50}) when administered intravaneously or intraperitoneally. Most orally ingested bacteria (>99%) are killed in the stomach or passed out of the gut. However, some survive in the intestine, from where they can traverse the intestinal epithelium (Carter and Collins, 1974). Several features of murine intestinal pathology are similar to

that observed in typhoid patients: notably diffuse enteritis, enlarged PP and thickening of the ileal mucosa, accompanied by a characteristic mononuclear leukocyte infiltrate (Santos et al., 2001). This is followed by dissemination to and colonisation of the MLN, liver and spleen, mirroring the tissue distribution of bacteria in human typhoid. *S.* Typhimurium predominantly replicates in the liver and spleen, within host phagocytes (Richter-Dahlfors *et al.*, 1997, Salcedo *et al.*, 2001), and this is associated with the development of hepatosplenomegaly. Microscopic examination of infected organs reveals granulomatous lesions formed initially of PMNs and later mononuclear cells, with central areas of necrosis (Nakoneczna and Hsu, 1980, Nakoneczna and Hsu, 1983). Similar lesions have also been described in chimpanzees (Edsall *et al.*, 1960) and humans (Mert *et al.*, 2004).

In addition to causing acute infections, *S.* Typhi is also able to chronically persist within infected individuals for long periods of time. This syndrome cannot be appropriately studied in Slc11a1-deficient mice, due to their exquisite susceptibility to infection with *S.* Typhimurium. However, Slc11a1-resistant mice routinely develop sublethal infections when infected with virulent *S.* Typhimurium and can harbour persistent reservoirs of these bacteria within infected tissues for up to one year (Monack *et al.*, 2004a). Therefore, this adapted model has since been used to study the mechanisms underlying chronic *S.* Typhi carriage.

Recently, two groups have developed humanised mouse models, in which S. Typhi is capable of causing a systemic disease. Immunodeficient alymphoid recombination-activating gene-2$^{-/-}$ interleukin (IL)-2 receptor gamma subunit (rγ)$^{-/-}$ (Song *et al.*, 2010) and nonobese diabetic-severe combined immunodeficient IL-2rγ$^{-/-}$ mice (Libby *et al.*, 2010) are partly humanised by engraftment with human hematopoietic stem cells. As a result, mice develop human-derived phagocytic cells that can then serve as a niche for S. Typhi infection. At present, these models are unlikely to be used for routine laboratory studies as the humanised mice display varying infection outcomes and are labour intensive to generate. However, these animals could prove useful for pre-clinical testing of vaccine candidates.

1.3.3. Limitations of animal models

The murine typhoid model has been used for decades to provide very detailed information on bacterial virulence and host defence mechanisms that are directly relevant to human typhoid fever. However, it is important to bear in mind that findings in the murine model do not necessarily directly translate to human infection. This is highlighted by a clear difference in the cause of death between murine and human typhoid. In humans, late stages of disease are characterised by re-seeding of bacteria from an infected gallbladder into the intestine, perforation of the gut and peritonitis (Bos and Willemsen, 2002). Although *S.* Typhimurium does colonise the gallbladder of mice (Monack *et al.*, 2004a, Menendez *et al.*, 2009), lethal infection of mice typically occurs as a result of large bacterial loads in the liver and spleen, leading to tissue damage, multi-organ failure and bacteraemia. In addition, mice infected with *S.* Typhimurium do not develop diarrhoea (Santos *et al.*, 2001), which is a common feature of human typhoid (Parry, 2006).

Discrepencies between the pathology of murine and human typhoid are likely to partly result from genetic differences in the development, activation and activity of immune cells between mice and man (Mestas and Hughes, 2004). In addition, significant differences exist between the genomes of *S.* Typhi and *S.* Typhimurium that also contribute to the altered disease outcomes in the two mammalian species. A greater understanding of this was made possible through the availability of the genome sequences of *S.* Typhi CT18 (Parkhill *et al.*, 2001) and *S.* Typhimurium LT2 (McClelland *et al.*, 2001). At least 204 genes present on the *S.* Typhimurium chromosome are absent from or functionally altered in *S.* Typhi (Parkhill et al., 2001). Of these pseudogenes, 145 represent intact genes in *S.* Typhimurium and up to 46 genes may contribute significantly to systemic virulence in the mouse model, including: fimbrial genes (Townsend *et al.*, 2001), effector genes of the T3SSs such as *sopD2* (Brumell *et al.*, 2003, Jiang *et al.*, 2004) and *sopE2* (Bakshi *et al.*, 2000) and the *Salmonella* plasmid virulence (*spv*) genes contained in the *Salmonella* virulence plasmid (pSLT) that is not carried by *S.* Typhi (Gulig *et al.*, 1992). Indeed, this genetic degradation is thought to explain the severe

host restriction of *S.* Typhi. Furthermore, the murine model cannot account for the function of the 601 genes that are unique to *S.* Typhi (Parkhill *et al.*, 2001) and which may contribute to its human tropism. These include genes located within SPI-7 that are important for virulence in typhoid fever, such as: genes encoding a type IVB pilus required for adhesion to and invasion of human monocytes and epithelial cells (Zhang *et al.*, 2000, Pan *et al.*, 2005) and the *via*B locus that is responsible for the biosynthesis, translocation and regulation of the Vi exopoylsaccharide antigen or capsule (Virlogeux *et al.*, 1995). Interestingly, the Vi capsule has been shown to downregulate toll-like receptor (TLR)-4-mediated activation of macrophages, potentially by masking LPS (Wilson *et al.*, 2008). In addition, the *tviA* operon contained within the *via*B locus has been reported to reduce flagellin secretion and so, downregulate TLR-5-mediated release of IL-8 by intestinal epithelial cells (Winter *et al.*, 2008). Collectively, these responses act to reduce inflammation and PMN accumulation within the intestine. The Vi capsule has also recently been suggested to confer resistance to complement-mediated phagocytosis and killing (Wilson *et al.*, 2011). Therefore, it is thought that functions conferred by this capsule might also underly the different disease outcomes produced in man by infection with *S.* Typhi and *S.* Typhimurium. The presence of the capsule in *S.* Typhi might promote colonisation of systemic sites through evasion of immune detection, however without this advantage, large pro-inflammatory responses are initiated by *S.* Typhimurium in the human gut, which restricts bacteria to gastrointestinal tissues.

1.4. Mechanisms of host resistance to *S. enterica* infection

Most understanding of the immunology of *S. enterica* infections has been obtained by studying host responses during sublethal infections of mice. In this model, the immunological response to *S.* Typhimurium can be divided into four distinct phases (Fig. 1.2) (Mastroeni, 2002). These involve innate immune effector mechanisms to moderate and soon after, suppress bacterial growth and antigen-specific adaptive immune responses, to eventually clear the infection and establish protection against a secondary infection.

1.4.1. Innate immunity to *S. enterica*

The gut provides the primary defense against *S. enterica* and controls the numbers of viable bacteria that are able to establish an invasive infection. After oral ingestion, *S. enterica* must contend with the extreme acidity of gastric acid, peristalsis of the intestinal tract and a diverse range of antimicrobial molecules, including: defensins, cathelicidin antimicrobial peptide (AMP) and bactericidal/permeability-inducing protein (Salzman *et al.*, 2003, Dann and Eckmann, 2007). Resident gut microbial flora, namely members of the genus *Bacteroides*, may also provide a barrier to *S. enterica*, through the production of volatile fatty acids (Bohnhoff *et al.*, 1964). Indeed, mice pre-treated with streptomycin, which disrupts the intestinal microbiota, are more susceptible to infection (Bohnhoff and Miller, 1962).

The damage caused through invasion of the intestinal mucosa and replication within host tissues undoubtedly exposes *S. enterica* to the bloodstream. Complement can subsequently opsonise, although not lyse, *S. enterica* (Joiner *et al.*, 1982), which promotes uptake of bacteria by phagocytic cells present within host tissues. Importantly, mice deficient in the C1q complement component experience delayed kinetics of *S.* Typhimurium clearance from the blood and are more susceptible to infection (Warren *et al.*, 2002).

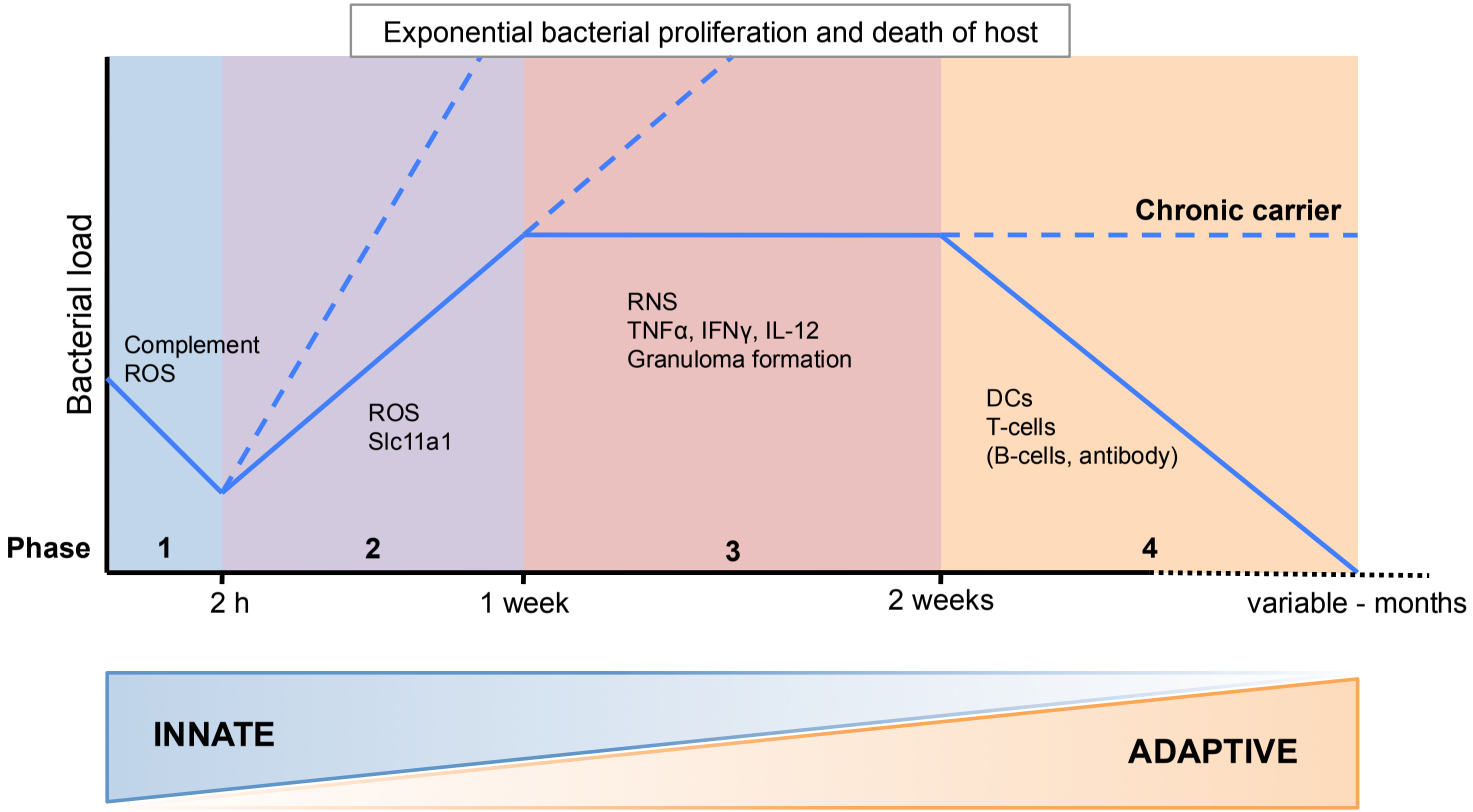


Figure 1.2. Immunology of a *S. enterica* infection. Four phases of a sub-lethal infection of *S. enterica* in immunocompetent mice (solid blue): (1) initial killing and clearance of bacteria from the bloodstream, involving complement and reactive oxygen species (ROS), (2) exponential intracellular bacterial net growth accompanied by ROS and Slc11a1-mediated bacterial killing and suppression of replication, respectively, (3) reactive nitrogen species (RNS)-mediated suppression of bacterial net growth, in addition to granuloma formation and cytokine production, (4) initiation of antigen-specific adaptive immunity leading to elimination of the infection (and prevention of a chronic carrier-state) and development of memory responses that can protect against re-infection. Dashed blue line indicates course of infection when immunological mechanisms at each stage are absent. Dendritic cells (DCs). Adapted from Mastroeni (2002).

Host phagocytes represent one of the most important components of innate immunity against *S. enterica*. Indeed, macrophages resident within host tissues are encountered by *S. enterica* soon after invasion. These are accompanied by the infiltration of PMNs within the first few days of infection and later, mononuclear cells (Nakoneczna and Hsu, 1980). Both macrophages and neutrophils have been implicated in the early control of *S. enterica* growth during infection (O'Brien *et al.*, 1979, Conlan, 1996, Conlan, 1997). Host phagocytes possess a diverse repertoire of pattern-recognition receptors (PRRs), which recognise conserved pathogen-associated microbial peptides, and can promote phagocytosis and upregulate phagocyte antimicrobial activities (Blander and Medzhitov, 2004). TLR-4 represents one well characterised PRR that plays an important role in the immunity against *S. enterica*. TLR-4 recognises LPS present in the outer membrane of *S. enterica* and this interaction induces macrophage activation by triggering signalling cascades that stimulate the release of nitric oxide and tumour-necrosis factor-alpha (TNFα) (Royle *et al.*, 2003). The importance of this function is highlighted by the finding that TLR-4-deficient mice rapidly succumb to infection with *S.* Typhimurium (O'Brien *et al.*, 1980), most likely due to delayed chemokine expression, inadequate macrophage function and impaired adaptive immune responses (Vazquez-Torres *et al.*, 2004, Weiss *et al.*, 2004).

The interaction of *S.* Typhimurium with host macrophages has been extensively studied. These immune cells employ several mechanisms to control the early growth of these bacteria. One key mediator is Slc11a1, which is present on the phagosome membrane in macrophages and localises to the *Salmonella*-containing vacuole (SCV) (Cuellar-Mata *et al.*, 2002). This metal transporter has a pleiotropic effect on innate resistance to *S. enterica*, including: limiting the availability of divalent cations to intracellular bacteria (Cellier *et al.*, 2007), promoting SCV fusion with degredative compartments (Cuellar-Mata *et al.*, 2002) and increasing macrophage antimicrobial activity, such as nitric oxide production (Ables *et al.*, 2001). Interestingly, recent evidence suggests that Slc11a1 also enhances host cell cytokine secretion and consequently, may influence the intensity of the inflammatory response

(Valdez *et al.*, 2008, Valdez et al., 2009). The activity of Slc11a1 seems to be involved in suppressing the early intracellular replication of *S.* Typhimurium (Hormaeche, 1980, Benjamin *et al.*, 1990) and it is well known that mice carrying a non-functional Slc11a1 protein are highly susceptible to *S.* Typhimurium infection (Vidal *et al.*, 1995). However, despite the important functions of this protein, no association between human Slc11a1 polymorphisms and typhoid fever has yet been found (Dunstan *et al.*, 2001a).

Macrophages also use a range of oxygen-dependent mechanisms to control *S. enterica* growth. Reactive oxygen species (ROS) are generated from the reduction of molecular oxygen by the multicomponent nicotinamide adenine dinucleotide phosphate-oxidase or phagocyte oxidase (Phox) (Vazquez-Torres and Fang, 2001). These free radicals are highly toxic to *S.* Typhimurium, although their precise targets still remain elusive (Slauch, 2011). Mice that lack a functional oxidative burst succumb to *S.* Typhimurium infection within the first few days of inoculation (De Groote *et al.*, 1997, Shiloh *et al.*, 1999, Mastroeni *et al.*, 2000b), resulting from little or no bacterial killing and increased bacterial replication (Grant *et al.*, 2008). Similarly, humans with chronic granulomatous disease, due to mutations in genes encoding Phox subunits, are predisposed to recurrent fungal and bacterial infections, including *S. enterica* (Mouy *et al.*, 1989). Infected macrophages also produce reactive nitrogen species (RNS), primarily through the oxidation of L-arginine by inducible nitric oxide synthase (iNOS) (Vazquez-Torres and Fang, 2001). In contrast to Phox-mediated killing, RNS are predominantly bacteriostatic and exert their function at later stages of the infection (Mastroeni *et al.*, 2000b). Mice deficient in iNOS are able to control the initial replication of *S.* Typhimurium, but develop fatal bacterial loads during the second and third weeks of infection (Shiloh *et al.*, 1999, Mastroeni *et al.*, 2000b). Additional mediators of phagocytic host resistance to *S. enterica* include hydrolytic enzymes and AMPs, such as cathelicidin-related AMP (Groisman *et al.*, 1992, Rosenberger *et al.*, 2004), although less is known about their functions.

As the infection progresses, multicellular focal lesions or granulomas, containing host phagocytes, gradually develop around infected cells. Immune

cell recruitment to and function within infection foci is directed by the release of cytokines, in particular: TNFα, IL-12 and interferon-gamma (IFNγ). Mice that lack these cytokines, due to gene deletions or antibody targeting, experience exacerbated infections with *S.* Typhimurium, particularly following the first week of infection (Muotiala and Makela, 1990, Mastroeni *et al.*, 1991, Mastroeni *et al.*, 1995, Everest *et al.*, 1998, Mastroeni *et al.*, 1998). Furthermore, humans with genetic or acquired deficiencies in the production of these cytokines are more susceptible to *S. enterica* infection (van de Vosse *et al.*, 2004). Therefore, appropriate granuloma formation and function is essential to control *S. enterica* growth.

Dendritic cells (DCs) play a crucial role in integrating innate and adaptive immune responses against *S. enterica*. Immature DCs are positioned within peripheral and lymphoid tissues and represent some of the first immune cells encountered by *S. enterica* within the host (Niess *et al.*, 2005). During murine typhoid, DCs rapidly undergo maturation within lymphoid tissues (Salazar-Gonzalez *et al.*, 2006). At these sites, mature DCs present bacterial antigens to adaptive immune cells, which can subsequently generate an antigen-specific response against the pathogen. Indeed, studies have demonstrated that DCs isolated from the spleen and liver can produce clonal expansion of *S. enterica*-specific cluster of differentiation factor (CD)4^+ and $CD8^+$ T cells *ex vivo* (Yrlid and Wick, 2002, Johansson and Wick, 2004). Furthermore, conditional DC ablation in transgenic mice abolished clonal expansion of *S.* Typhimurium-specific $CD4^+$ T-cells (Salazar-Gonzalez *et al.*, 2006).

1.4.2. Adaptive immunity to *S. enterica*

Although $CD4^+$ and $CD8^+$ T-cell depleted mice are able to control the early growth of *S. enterica*, these mice are unable to successfully eliminate bacteria or generate protective immunty against a subsequent infection (Hess *et al.*, 1996). This demonstrates the need for antigen-specific adaptive immunity. Importantly, human polymorphisms in major histocompatibility complex (MHC) class II genes have been linked to increased susceptibility to typhoid fever (Dunstan *et al.*, 2001b). Furthermore, it is well documented that HIV-infected

individuals, that develop significant impairment of T-cell-mediated immunity, are at increased risk of *S. enterica* infection (Gordon, 2008).

$CD4^+$ T-cells with a T helper type (Th)1 phenotype lie at the heart of the adaptive immune response (Hess *et al.*, 1996). The importance of Th1-defined T-cell populations is demonstrated by the increased susceptibility of mice deficient in T-bet, a transcription factor required for Th1 differentiation, to infection with *S.* Typhimurium (Ravindran *et al.*, 2005). Cross-presentation of *S. enterica* antigens on MHC class I can also induce $CD8^+$ T-cell responses *ex vivo* (Yrlid and Wick, 2002, Johansson and Wick, 2004). Indeed, *S. enterica*-specific $CD8^+$ T-cells have been isolated from humans immunised with attenuated *S.* Typhi vaccine strains (Sztein *et al.*, 1995). However, compared to $CD4^+$ T-cells, $CD8^+$ T-cells seem to play a smaller role (Hess *et al.*, 1996). The mechanisms used by T-cells to control *S. enterica* infection are still not well understood. It is likely that $CD4^+$ T-cells upregulate the microbicidal activities of macrophages and enhance appropriate antibody responses (Dougan *et al.*, 2011). In turn, innate immune cells also promote the development of efficient adaptive immunity, through the production of Th1-like cytokines, such as IFNγ and TNFα (Kirby *et al.*, 2002, Rydstrom and Wick, 2007). This creates a positive feedback loop that further amplifies both arms of the immune system, to effectively clear the infection.

In addition to T-cells, infection with *S. enterica* in mice and humans also generates bacteria-specific antibodies (Mastroeni, 2002), which indicates that salmonellae must be transiently exposed to the extracellular space during infection. Although B-cells seem dispensable for clearance of a primary infection, they play an important role in the acquired immune resistance to *S. enterica* (Mastroeni *et al.*, 2000a). Adoptive transfer experiments showed that full protection to virulent *S.* Typhimurium in previously susceptible mice is only conferred after the simultaneous administration of both immune serum and cells from vaccinated mice (Mastroeni *et al.*, 1993). Antibody may contribute to killing of *S. enterica* by promoting phagocytosis of bacteria and upregulating oxidative responses within innate phagocytic cells (Uppington *et*

al., 2006). In addition, B-cells can influence the T-cell arm of the immune response, by presenting antigens to $CD4^+$ T-cells. The importance of this pathway was demonstrated by the inability of B-cell-deficient mice to generate Th1 T-cell immunity needed to clear a secondary infection of *S.* Typhimurium (Mastroeni *et al.*, 2000a). Indeed several human immunodeficiency syndromes, characterised by defects in both B- and T-cell-mediated responses, have been reported to increase the risk of salmonellosis (Dougan *et al.*, 2011). This demonstrates that both cellular and humoral immune effector mechanisms are required to effectively protect against a secondary infection with *S. enterica.* However, it is important to bear in mind that even after a natural infection of humans with *S.* Typhi, a robust immune response is not always generated, since patients can relapse or become chronic carriers.

In conclusion, complex mechanisms involving cross-talk between innate and adaptive immune cells and integration of T- and B-cell-mediated responses are required to establish an effective immunological response against *S. enterica*. The mouse model has proven invaluable for dissecting some of these responses, particularly the use of gene knockout mouse strains, and many findings in mice have correlated with similar observations in humans. However, further studies using this model, with an emphasis on translation to human typhoid, are needed to provide a more complete understanding of *S. enterica* immunity and this will no doubt facilitate the development of more efficient vaccines against typhoid fever.

1.5. *S. enterica* virulence factors

1.5.1. The *S.* Typhimurium genome

Salmonellae have evolved into very succesful pathogens as a result of horizontal gene transfer. This is a process that can rapidly alter microbial genomes through the acquisition of large segments of DNA, known as genomic islands, from unrelated organisms via transduction, transformation or conjugation. Genomic islands characteristically have a base composition, determined by the percentage of guanine and cytosine residues (% G + C), that is different from that of the host core genome. They are typically found within or adjacent to genetic elements involved in DNA mobility, such as plasmids, phages, transposases, integrases and direct repeat sequences. In addition, DNA insertion sites are frequently associated with genes encoding transfer ribonucleic acids (tRNAs), as these can act as chromosomal integration points for certain bacteriophages. Many genes involved in bacterial virulence are located to clusters of horizontally acquired DNA and in these cases, such regions are referred to as pathogenicity islands (PAIs) (Hensel, 2006).

Genomic sequence analysis of *S.* Typhimurium LT2 has revealed that this serovar has a mosaic genome, which has been greatly influenced by horizontal gene transfer. 29% of genes in *S.* Typhimurium are absent in the non-pathogenic ancestor *Escherichia coli* (*E. coli*) (McClelland *et al.*, 2001). The chromosome consists of 4,875 kilobases (kbs) and has a G + C content of 53% (McClelland *et al.*, 2001). Approximately 4% (200 genes), or more, of the genome is required for systemic disease in mice (Bowe *et al.*, 1998, Lawley *et al.*, 2006, Chaudhuri *et al.*, 2009). These genes are located within at least 12 PAIs, 4 prophages (Gifsy-1, Gifsy-2, Fels-1 and Fels-2) and within an approximately 90 kb extrachromosomal self-transmissible plasmid, pSLT (Sabbagh *et al.*, 2010, McClelland *et al.*, 2001). A diverse array of virulence determinants are encoded within these regions, which include regulatory systems, biosynthetic enzymes, nutrient transporters, adhesins, secretion systems and their effectors.

1.5.2. The PhoPQ regulon

S. enterica must survive a wide range of changing environmental conditions and antimicrobial defenses during the course of an infection within the host and of course, during transmission to new hosts. These include alterations in pH, osmolarity and oxygen tension and in addition, the presence of antimicrobial substances that are secreted in the intestine and produced within specific immune effector cells. To enable adaptation to these stressful environments, bacteria possess complex networks of two-component regulatory systems, transcriptional regulators and alternate sigma factors that control the spatiotemporal expression of many genes. One of the best characterised transcriptional regulons, that is important for *S. enterica* pathogenesis, is that controlled by the PhoP/PhoQ (PhoPQ) two-component regulatory system.

PhoPQ is a major transcriptional regulator in *Salmonella* and controls the transcription, positively or negatively, of more than 40 virulence genes of *S.* Typhimurium (Miller *et al.*, 1989, Groisman *et al.*, 1989, Miller and Mekalanos, 1990). Interestingly, only 25% of PhoPQ-modulated genes are involved in *S. enterica* virulence (Garcia Vescovi *et al.*, 1994). In addition, *phoP* homologues are present within numerous other pathogenic and non-pathogenic bacteria. Together, this suggests that PhoPQ represents an ancient system that served to regulate genes unrelated to virulence, such as those required for survival in the environment. However, selective pressure experienced by *S. enterica* has caused this system to evolve to enable these bacteria to respond to conditions experienced within living hosts.

Although the signals regulating PhoPQ *in vivo* have not been fully defined, activation can be induced *in vitro* under several conditions, including: acidic pH, low concentration of divalent cations (Mg^{2+} and Ca^{2+}), and in the presence of certain AMPs (Alpuche-Aranda *et al.*, 1992, Vescovi *et al.*, 1996, Bader *et al.*, 2005). The kinase, PhoQ, can sense these environmental signals and respond by phosphorylating the transcriptional activator, PhoP (Gunn *et al.*, 1996). PhoPQ is fully activated several hours following uptake by host

macrophages, as bacteria become enclosed within an acidic, nutrient limiting phagosome that is rich in AMPs (Alpuche-Aranda *et al.*, 1992, Valdivia and Falkow, 1997). The expression of a diverse set of genes, termed *pags* (PhoP-activated genes), is activated by this regulon and these promote survival of *S. enterica* within host tissues. *Pags* are multifunctional and include enzymes, that modify LPS to confer resistance to AMP-mediated killing, cation transporters, outer membrane proteins and genes involved in acid tolerance (Prost *et al.*, 2007). In addition, PhoP represses the transcription of other genes, namely those associated with the SPI-1 T3SS (see below) and flagella that are required for invasion and motility, respectively (Prost *et al.*, 2007). These are designated *prgs* (PhoP-repressed genes). The importance of PhoPQ is evident by the severe attenuation of a non-functional PhoP mutant of *S.* Typhimurium for replication and survival within macrophages *in vitro* and for systemic virulence in mice (Fields *et al.*, 1989, Miller *et al.*, 1989, Thompson *et al.*, 2011) and because a *phoPQ*-deleted *S.* Typhi strain was significantly attenuated in humans (Hohmann *et al.*, 1996). Deletions of individual or several *pags* does not significantly alter the virulence of *S.* Typhimurium (Gunn *et al.*, 1998), except for the MgtC mutant (Blanc-Potard and Groisman, 1997), suggesting a functional redundancy within these genes. Notably, a *S.* Typhimurium mutant with a constitutively activated PhoPQ system is also attenutated in mice, indicating that appropriate regulation of *pags* and *prgs* is also an important virulence trait (Miller and Mekalanos, 1990).

1.5.3. Type 3 secretion systems

T3SSs are widespread among pathogenic Gram-negative bacteria and provide a mechanism to enable the delivery of bacterial proteins or effectors directly into the host cell cytosol and membranes, to subvert host cell functions (Hueck, 1998). The secretion or injectisome machinery is highly conserved and resembles flagellar export apparatus, suggesting they evolved from a common ancestor. Approximately 20 proteins assemble to form a basal body that spans the inner and outer bacterial membranes, a needle-like hollow tube that extends from the bacterial surface and a translocon or pore

that inserts into the host cell membrane. Effector translocation is driven by an adenosine triphosphatase (Eichelberg *et al.*, 1994). In addition, several chaperones help to maintain effector proteins in an unfolded or partially-folded conformation for efficient secretion (Stebbins and Galan, 2001, Luo *et al.*, 2001). Although components of the secretion apparatus are highly conserved, the effector proteins are diverse. *Salmonella enterica* is unusual in that it expresses two separate T3SSs. These are the SPI-1 and SPI-2 T3SSs, which are important for invasion of host cells (Galan *et al.*, 1989) and intracellular replication (Ochman *et al.*, 1996), respectively. Since genes within SPI-1 are only distantly related to those of SPI-2 (Shea *et al.*, 1996) and only SPI-1 is present in *S. bongori* (Hensel *et al.*, 1997), this suggests that both PAIs were acquired as independent horizontal transfer events. Interestingly, the expression of both secretion systems is controlled by local and global regulators (Garmendia *et al.*, 2003, Altier, 2005, Fass and Groisman, 2009), which suggests that integration into existing regulatory pathways has presumably evolved, enabling the expression of these virulence genes to be controlled in a coordinate and appropriate manner.

1.5.3.1. The SPI-1 T3SS

SPI-1 represents a 40 kb large cluster of genes situated within centisome 63 of the *S. enterica* chromosome (Mills *et al.*, 1995). Although not associated with a tRNA gene, the SPI-1 locus has a G + C content of 47%, significantly lower than the core *S. enterica* genome. This PAI encodes the structural, effector and regulatory proteins of the SPI-1 T3SS (Darwin and Miller, 1999), which has been directly visualised as a rigid, hollow tube on the surface of *S.* Typhimurium by electron microscopy (Kubori *et al.*, 1998). The transcriptional regulator HilA plays a central role in controlling SPI-1 gene expression (Altier, 2005). Activation of this virulence system is induced by high osmolarity, low oxygen, near-neutral pH and in the presence of the short-chain fatty acid, acetate (Altier, 2005). These environmental signals are likely to be found in the distal ileum, where the SPI-1 T3SS mediates invasion of the intestinal epithelium.

At least 15 effector proteins are translocated into the host cell by the SPI-1 T3SS and these include effectors encoded within and outside SPI-1 (McGhie *et al.*, 2009). A major function of the SPI-1 T3SS is to induce bacterial internalisation into non-phagocytic mammalian cells and mutants that do not express a functional SPI-1 T3SS are attenuated approximately 50-fold following oral inoculation of mice (Galan *et al.*, 1989). This demonstrates that the invasive properties conferred by the SPI-1 T3SS contribute to efficient penetration of the intestinal epithelium and establishment of a systemic infection.

Upon translocation, SipA, SipC, SopB, SopD, SopE and SopE2 either directly or indirectly induce the spatiotemporal rearrangement of the actin cytoskeleton (Fig. 1.3). SipC is a translocon component of the SPI-1 T3SS and inserts into the host cell plasma membrane (Scherer *et al.*, 2000). Within the host cell, SipC stimulates filamentous actin (F-actin) polymerisation, by nucleating globular actin, and subsequently bundles the actin filaments (Hayward and Koronakis, 1999). SipA enhances the activity of SipC, by binding to and stablising acting filaments and inhibiting their depolymerisation (Zhou *et al.*, 1999, McGhie *et al.*, 2001, McGhie *et al.*, 2004). The activity of both SipA and SipC is thought to direct the spatial localisation of actin polymerisation underneath the bacteria. SopE and SopE2 are homologues that mimic cellular guanidine nucleotide exchange factors (GEFs) and activate Rho guanine nucleotide triphosphatases (GTPases), including Rac-1 and Cdc-42 (Wood *et al.*, 1996, Hardt *et al.*, 1998, Bakshi *et al.*, 2000). However, SopE-dependent Rac-1 activation is most important for bacterial invasion (Patel and Galan, 2006). SopB is an inositol phosphatase, encoded within SPI-5, that is likely to alter cellular phosphoinositide phosphate and inositol phosphate metabolism (Norris *et al.*, 1998). Through this activity, SopB indirectly activates Cdc-42 (Zhou *et al.*, 2001) and another GTPase, RhoG (Patel *et al.*, 2006), the latter mediated by stimulation of an endogenous SH3-containing GEF. Activation of GTPases is essential for internalisation of *S. enterica*, as a triple SopE/E2/B mutant is completely defective for invasion (Zhou *et al.*, 2001). Together, these activated GTPases initiate complex signalling events that lead to actin nucleation, polymerisation, branching and

cross-linking, which induces ruffling and extension of the host cell membrane to engulf the bacterium (Patel and Galan, 2005). Following invasion, SopB, together with SopD, enhance membrane fission and promote macropinocytosis (Hernandez *et al.*, 2004, Bakowski *et al.*, 2007). Downregulation of actin remodelling occurs shortly after and is mediated by the GTPase activating protein (GAP) activity of SptP, which downregulates Cdc-42 and Rac-1 (Fu and Galan, 1999).

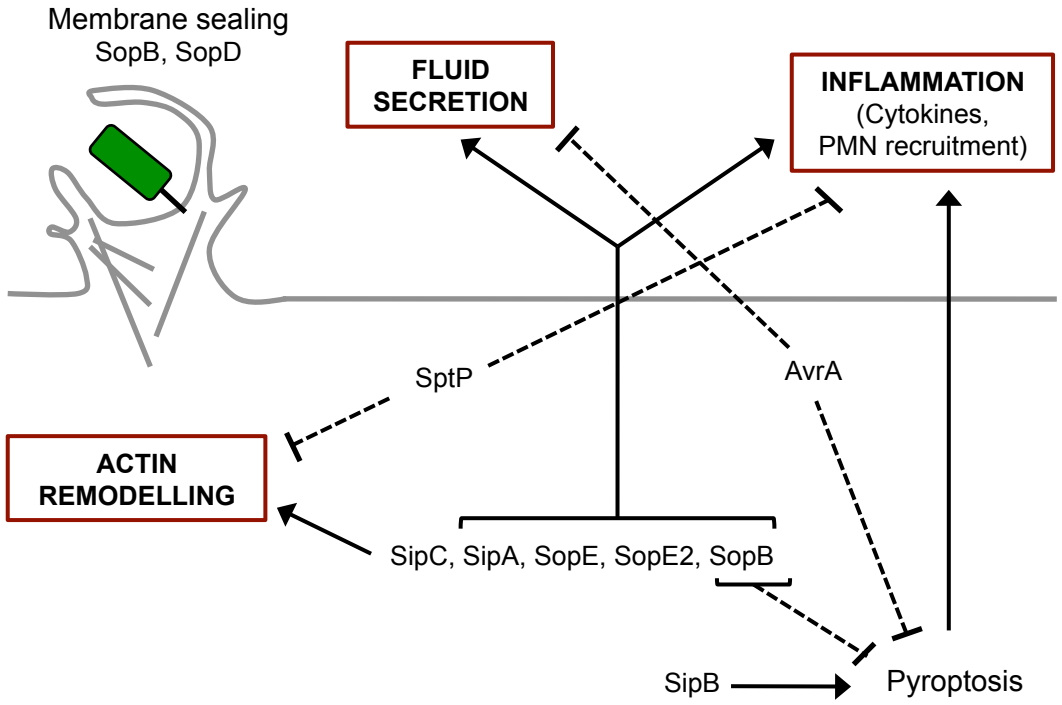


Figure 1.3. Functions of SPI-1 T3SS effectors. SipC, SipA, SopE, SopE2 and SopB are involved in actin rearrangements that lead to uptake of *S.* Typhimurium by non-phagocytic cells. These events are terminated by SopB and SopD, which contribute to membrane sealing, and SptP, which downregulates actin rearrangements. Several effectors also promote intestinal inflammation and fluid secretion, including SipA, SopE, SopE2 and SopB. This inflammation is enhanced by SipB-induced pyroptosis. Other effectors, namely SptP and AvrA act to downregulate these responses.

Another prominent function of the SPI-1 T3SS is the generation of a potent inflammatory response, characterised by PMN transmigration into the intestinal lumen, which is a hallmark of *S. enterica*-induced colitis. This is likely to be mediated by several mechanisms (Fig. 1.3). SipA has been shown to trigger the release of pathogen-elicited epithelial chemoattractant, now

known as hepoxillin A3, by epithelial cells (Lee *et al.*, 2000, Criss *et al.*, 2001, Mrsny *et al.*, 2004). Other effectors, SopE, SopE2 and SopB, mediate the activation of Cdc-42, which upregulates mitogen-activated protein kinase (MAPK) pathways and activates nuclear factor-kappa beta (NF-κB) signalling within epithelial cells (Galyov *et al.*, 1997, Patel and Galan, 2006, Bruno *et al.*, 2009). This stimulates the release of proinflammatory cytokines, such as IL-8 and macrophage inflammatory protein-2-alpha. Flagellin secretion appears to promote these responses (Gewirtz *et al.*, 2001, Huang *et al.*, 2004). In addition, SopA, an E3 ubiquitin ligase, has also been reported to promote PMN transmigration into the intestinal lumen (Wood *et al.*, 2000, Zhang *et al.*, 2006).

The SPI-1 T3SS also induces host cell death, although biological significance of this is still unclear. SPI-1-induced cytotoxicity involves the activity of the translocon component, SipB (Hersh *et al.*, 1999), and flagellin (Miao *et al.*, 2006, Franchi *et al.*, 2006), which produces rapid cytotoxicity in macrophages and DCs, termed pyroptosis (Chen *et al.*, 1996, Monack *et al.*, 1996, van der Velden *et al.*, 2003). SipB and flagellin activate caspase-1, leading to cleavage and activation of the pro-inflammatory cytokines IL-1β and IL-18, which promote local inflammatory responses (Fink and Cookson, 2007). SipB also produces a delayed caspase-1-independent form of cell death in infected macrophages (Hernandez *et al.*, 2003).

Other functions of SPI-1 effectors include the development of diarrhoea observed in NTS. SopB can activate epithelial cell chloride channels, through its inositol phosphatase activity, and promote fluid secretion into the intestinal lumen (Galyov *et al.*, 1997, Norris *et al.*, 1998). This effector, along with SopE, SopE2 and SipA, has also been shown to stimulate fluid secretion by disrupting epithelial tight junctions, which most likely occurs via Rho GTPase signalling (Boyle *et al.*, 2006). SopA is also likely to contribute to fluid secretion (Wood *et al.*, 2000).

Interestingly, the function of some SPI-1 effectors is to oppose the actions of other effectors, translocated by the same secretion system (Fig. 1.3). As

mentioned above, the GAP activity of SptP causes downregulation of actin cytoskeleton rearrangements. In addition, both GAP and tyrosine phosphatase activities displayed by SptP can reverse MAPK responses to reduce inflammation (Murli *et al.*, 2001), Another effector, AvrA, has been implicated in the suppression of multiple SPI-1 effector-mediated cellular responses. This includes the opposition of tight-junction disruption, leading to a reduction of epithelial cell permeability and intestinal fluid accumulation (Liao *et al.*, 2008) and inhibition of apoptosis, through acetyltransferase-mediated blockade of MAPK kinase activation (Jones *et al.*, 2008). SopB has also been shown to inhibit apoptosis of host cells (Knodler *et al.*, 2005b). Collectively, these findings demonstrate the multifunctional capabilities of the SPI-1 T3SS-secreted effectors and highlight the complex nature of the host responses that they regulate.

1.5.3.2. The SPI-2 T3SS

SPI-2 occupies 40 kb at centisome 30 on the *S.* Typhimurium chromosome and lies adjacent to a tRNA gene, *valV* (Hensel *et al*., 1995, Hensel *et al.*, 1997). Genes encoding the secretion system are located within a 26 kb region towards one end of the island that has a G + C content of 43%, significantly lower than that of the core genome. The major direct regulator of SPI-2 gene expression is the SsrAB two-component system, encoded within the SPI-2 locus (Valdivia and Falkow, 1997, Cirillo *et al.*, 1998). Activation of SPI-2 occurs intracellularly, within the SCV (Valdivia *et al*., 1997, Cirillo *et al*., 1998); a specialised compartment that *S. enterica* replicates within once inside host cells. However, SPI-2 has also been shown to be expressed within the intestinal lumen (Brown *et al.*, 2005). Nutrient limitation within the SCV lumen is likely to trigger expression of the SPI-2 genes (Deiwick *et al.*, 1999). Acidification of this compartment also activates SPI-2 genes (Cirillo *et al.*, 1998) and is important for assembly of the secretion apparatus (Beuzon *et al.*, 1999, Rappl *et al.*, 2003). Effector translocation subsequently occurs once translocon pore formation exposes the bacterium to the neutral pH of the host cell cytosol (Yu *et al.*, 2010).

SPI-2 T3SS-deficient *S.* Typhimurium mutants have replication defects within epithelial cells and macrophages (Ochman *et al.*, 1996, Cirillo *et al.*, 1998, Hensel *et al.*, 1998) and are significantly attenuated in mice, following oral or i.p. inoculation (Hensel *et al.*, 1995, Shea *et al.*, 1996). This correlates with an inability to colonise the MLN, liver and spleen (Cirillo *et al.*, 1998, Shea *et al.*, 1999). However, the survival of *S.* Typhi within human monocyte-like cells has been shown to be independent of the SPI-2 T3SS (Forest *et al.*, 2010). Over 20 effectors, encoded within SPI-2, pSLT or elsewhere in the chromosome, have been shown to require the SPI-2 T3SS for their translocation (McGhie *et al.*, 2009), although only the function of a few of these proteins has so far been identified. The major role of the SPI-2 effectors is to support intracellular bacterial replication (Helaine *et al.*, 2010), which involves manipulation of vesicular trafficking and signalling pathways.

During infection of epithelial cells, the SCV gradually migrates from the periphery of the host cell towards a perinuclear region (Salcedo and Holden, 2003). It is thought that this facilitates interactions with secretory vesicles of the nearby Golgi, which may provide nutrients and/or membrane to support bacterial replication. Retention of the SCV at a juxtanuclear position is necessary for efficient replication and requires the concerted action of the SPI-2 T3SS effectors, SseF and SseG (Salcedo and Holden, 2003, Abrahams and Hensel, 2006) (Fig. 1.4). The precise mechanism underlying SCV positioning is not completely understood. Both recruitment of the minus end-directed microtubule motor, dynein, to the SCV (Abrahams *et al.*, 2006) and tethering of the SCV to the Golgi (Ramsden *et al.*, 2007) have been implicated.

Upon reaching a perinuclear position, *S.* Typhimurium begins to replicate and in epithelial cells, this is accompanied by the formation of *Salmonella*-induced filaments (Sifs), which radiate from the microcolony towards the periphery of the cell (Garcia-del Portillo *et al.*, 1993). These are lysosomal membrane glycoprotein-rich tubules that are closely associated with the microtubule network and are thought to represent the outcome of successive late endosome fusion events with the SCV. SifA is essential for the formation of

Sifs (Stein *et al.*, 1996) and this activity is augmented by the actions of other effectors, including SseG, SseF, PipB2 and SopD2 (Guy *et al.*, 2000, Jiang *et al.*, 2004, Knodler and Steele-Mortimer, 2005) (Fig. 1.4). In contrast, SseJ has been suggested to oppose Sif formation (Ruiz-Albert *et al.*, 2002). Interestingly, recent work has revealed the presence of additional tubules containing Golgi-derived secretory carrier membrane protein-3 (Mota *et al.*, 2009) or sorting nexin-3 (Braun *et al.*, 2010). The induction of tubule networks by *S.* Typhimurium is intriguing and the physiological relevance of these structures is still not clear. They may facilitate nutrient and/or membrane acquisition, direct harmful molecules away from the SCV or represent a means that bacteria deal with dynein and kinesin tension on the SCV. Alternatively, they could be an epiphenomenon of no physiological importance. However, mutants that lack any of the effectors involved in SCV tubulation are attenuated in mice (Hensel *et al.*, 1998, Beuzon *et al.*, 2000, Knodler *et al.*, 2003, Jiang *et al.*, 2004).

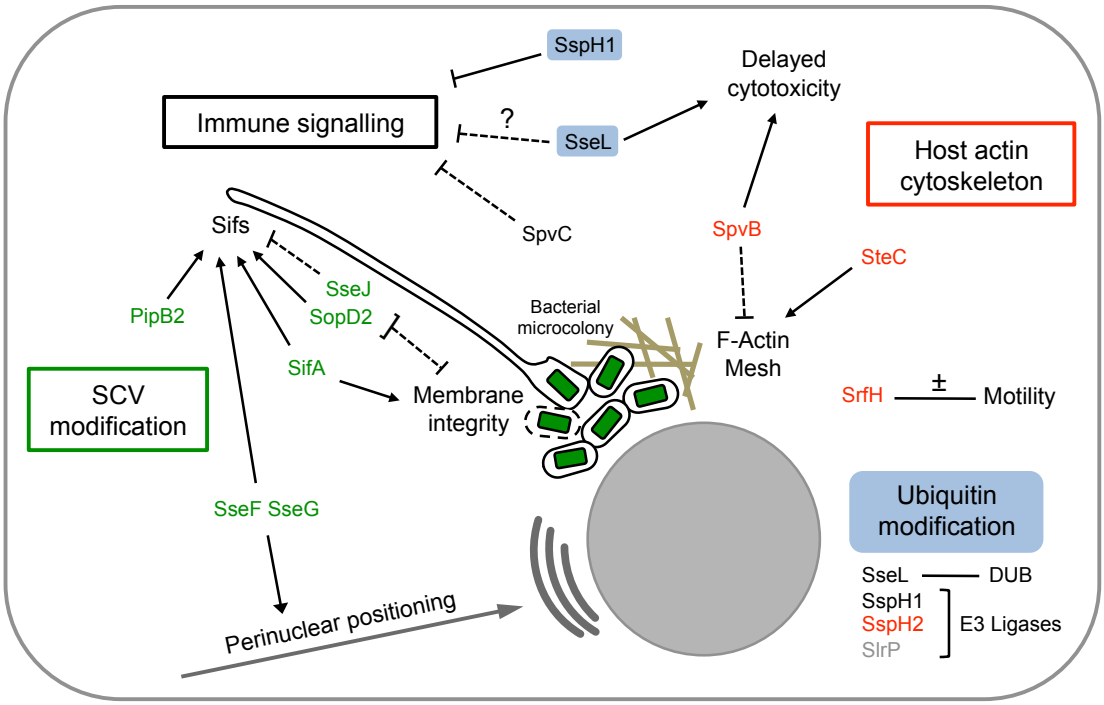


Figure 1.4. Functions of the SPI-2 T3SS effectors. The main functions of SPI-2 T3SS-associated effectors include modification of the SCV membrane and position (green box), downregulation of immune signalling (black box), interference with the host actin cytoskeleton (red box) and potentially modification of cellular ubiquitin (blue box). The effectors involved in each aspect of SPI-2 T3SS function are coloured accordingly. DUB (deubiquitinase)

S. Typhimurium strains that lack SifA lose their vacuolar membrane and display a profound growth defect within macrophages and in mice (Beuzon *et al.*, 2000). This provides a clear indication that SifA has a crucial role in maintaining the stability of the vacuolar membrane (Fig. 1.4). Interestingly, a *sifA sseJ* double mutant is retained within an intact vacuole, suggesting that SseJ, a glycerophospholipid:cholesterol acyltransferase, is involved in the loss of SCV integrity (Ruiz-Albert *et al.*, 2002). SopD2 also appears to contribute to vacuolar membrane loss (Schroeder *et al.*, 2010).

Another phenonemon associated with the SPI-2 T3SS activity is the assembly of a F-actin meshwork around the SCV (Méresse *et al.*, 2001). This is promoted by the kinase, SteC (Poh *et al.*, 2008), and inhibited by SpvB (Miao *et al.*, 2003) (Fig. 1.4). However, the function of actin meshwork formation is unclear, given that a SteC mutant does not appear to exhibit a virulence defect in mice (Geddes *et al.*, 2005, Poh et al., 2008).

Another effect associated with the SPI-2 T3SS is host cell death (van der Velden *et al.*, 2000) (Fig. 1.4). This is observed *in vitro* as a delayed form of apoptosis in epithelial cells and macrophages that involves caspase-3 and possibly, caspase-1 (Monack *et al.*, 2001, Paesold *et al.*, 2002). SPI-2-dependent cytotoxicity is mediated by the adenosine diphosphate-ribosylase activity of SpvB, encoded on the *spv* operon, which causes actin depolymerisation and destabilisation of the eukaryotic actin cytoskeleton (Libby *et al.*, 2000, Lesnick *et al.*, 2001, Browne *et al.*, 2002, Browne *et al.*, 2008). The deubiquitinase, SseL, also seems to contribute to this cytotoxic effect (Rytkonen *et al.*, 2007), although the mechanistic basis underlying this remains undefined. The importance of delayed SPI-2-dependent cytotoxicity to *S. enterica* pathogenesis requires further clarification, however it is possible that it might contribute to bacterial spreading within host tissues (discussed in Section 1.6.2).

Another effector, SrfH (also known as SseI), appears to influence bacterial movements *in vivo*. One group has reported that infected phagocytes display increased dissemination away from the intestinal lumen to the liver and

spleen, in an SrfH-dependent manner (Worley *et al.*, 2006). Therefore, the authors argue that SrfH promotes the development of a systemic infection. However, another study suggested that SrfH inhibits the motility of infected phagocytes to the spleen and therefore, promotes the establishment of a persistent infection (McLaughlin *et al.*, 2009). Further work is required to explain these apparently contradictory results. Nevertheless, the possibility that *S. enterica* may influence the movement of host cells to enhance bacterial dissemination or persistence *in vivo* presents an exciting and novel area of *Salmonella* research.

Several SPI-2 effectors also seem to be involved in downregulating cellular immune responses (Fig. 1.4). SpvC displays phosphothreonine lyase activity and irreversibly inhibits MAPK signalling (Li *et al.*, 2007, Mazurkiewicz *et al.*, 2008). In addition, the E3 ubiquitin ligase, SspH1 (Rohde *et al.*, 2007) and the deubiquitinase, SseL (Coombes *et al.*, 2007, Rytkonen *et al.*, 2007), have been suggested to inhibit NF-κB activity, possibly by modulating the ubiquitination of host proteins (Haraga and Miller, 2003, Haraga and Miller, 2006, Le Negrate *et al.*, 2008). However, conflicting results for SseL were obtained by our group (Rytkonen *et al.*, 2007). SspH2 and SlrP have also been identified as E3 ubiquitin ligases (Quezada *et al.*, 2009, Bernal-Bayard and Ramos-Morales, 2009), although their functions are yet to be determined.

Other functions of the SPI-2 T3SS include avoidance of Phox (Mastroeni *et al.*, 2000b, Gallois *et al.*, 2001), although this has been challenged recently (Helaine et al., 2010, Aussel *et al.*, 2011), and iNOS activity (Chakravortty *et al.*, 2002), conferring resistance to oxidative and nitrosative stress, respectively. In addition, SPI-2 T3SS-associated effectors have been reported to inhibit antigen presentation (Mitchell *et al.*, 2004, Cheminay *et al.*, 2005), by interfering with peptide loading on to MHC class II complexes (Halici *et al.*, 2008) and/or inducing polyubiquitination-mediated removal of peptide loaded MHC class II complexes from the host cell surface (Lapaque *et al.*, 2009). However, the molecular mechanisms and effectors involved in these processes are not yet fully understood.

Therefore, the SPI-2 T3SS delivers effectors into host cells, which are able to interfere with a diverse range of cellular processes. However, much work is required to provide a more complete understanding of this virulence system to *S. enteria* pathogenesis *in vivo*. Lastly, it is important to mention that several SPI-1-translocated effectors have been implicated in SCV biogenesis, positioning, membrane integrity and interference with endosomal trafficking pathways (McGhie *et al.*, 2009). Furthermore, certain SPI-1 T3SS-associated proteins have been shown to be expressed in the MLN and spleens of mice after several days of infection (Giacomodonato *et al.*, 2007, Gong *et al.*, 2009). Recently, translocation of SopB in the MLN of mice has even been demonstrated (Giacomodonato *et al.*, 2011) and some SPI-1 genes have also been implicated in long-term systemic infection in mice (Lawley *et al.*, 2006). However, set against this are data showing that SPI-1 T3SS-null mutants display no apparent virulence defect following intraperitoneal (i.p.) inoculation of mice (Galan *et al.*, 1989). Nevertheless, several lines of evidence now exist, which challenge the view that the SPI-1 and SPI-2 secretion systems represent functionally distinct virulence factors.

1.5.4. The *spv* operon

Many different *S. enterica* serovars contain plasmids that are associated with virulence, such as the 90 kb pSLT plasmid of *S.* Typhimurium (Gulig and Curtiss, 1987). This contains an approximately 8 kb region that contains the *spv* operon (Gulig *et al.*, 1993): *spvR*, which encodes a transcriptional regulator and four genes, *spvABCD*. *Spv* genes are required for systemic growth of *S.* Typhimurium in mice (Gulig and Doyle, 1993) and have also been implicated in invasive NTS infections in humans (Fierer *et al.*, 1992). Currently, functions have only been ascribed for SpvB and SpvC and both these proteins appear to be largely responsible for the *spv*-mediated virulence of *S.* Typhimurium in mice (Matsui *et al.*, 2001). These two effectors are translocated by the SPI-2 T3SS and their functions are described above.

1.6. Dynamics of growth and dissemination of *S. enterica* during systemic disease

1.6.1. Multiple routes of systemic dissemination

Following ingestion, *S.* Typhimurium uses several routes to traverse the intestinal barrier and gain access to systemic sites (Fig. 1.5). The primary site of intestinal invasion is in the distal ileum (Carter and Collins, 1974). Here, the SPI-1 T3SS enables bacteria to translocate an array of proteins into host cells to drive their own uptake. In this manner, *S.* Typhimurium invades both enterocytes and M cells enriched within the follicule-associated epithelium (FAE) that overlies the PP (Clark *et al.*, 1994, Jones *et al.*, 1994). Examination of infected ileal loops by electron microscopy (Jones *et al.*, 1994), together with the finding that a SPI-1 T3SS mutant is significantly attenuated in systemic virulence following oral inoculation (Galan *et al.*, 1989), demonstrates that SPI-1-directed invasion of M cells represents the predominant route of intestinal traversal. However, *S.* Typhimurium has also been shown to traverse the intestinal epithelium via PP-independent routes, including via $CD18^{+}$ phagocytes (Vazquez-Torres *et al.*, 1999), lamina propria DCs (Rescigno *et al.*, 2001, Niess *et al.*, 2005, Chieppa *et al.*, 2006), villous M-cells (Jang *et al.*, 2004) and M-cells within the FAE overlying solitary intestinal lymphoid tissues (Halle *et al.*, 2007). Infections carried out using SPI-1 T3SS mutants revealed that several of these PP-independent routes involve SPI-1 T3SS-independent mechanisms (Vazquez-Torres *et al.*, 1999, Niess *et al.*, 2005, Halle *et al.*, 2007).

Following invasion of the intestinal epithelium, presumably lymphatic drainage of the PP and/or lamina propria transports *S.* Typhimurium from these tissues to the MLN. However, it is not known how bacteria are transported in the lymph. In the MLN, the majority of *S.* Typhimurium-infected cells represent DCs (Voedisch *et al.*, 2009, Cheminay *et al.*, 2005). These cells might have migrated from the PP (Hopkins *et al.*, 2000) or lamina propria (Bogunovic *et al.*, 2009). Indeed, modulation of DC migration had a significant influence on colonisation of the MLN by *S.* Typhimurium (Voedisch *et al.*, 2009).

Furthermore, DCs are known to be important for transporting commensal bacteria to the MLN (Macpherson and Uhr, 2004). Therefore, DCs in the lymph could transport *S.* Typhimurium to the MLN from the PP and/or lamina propria (Fig. 1.5). However, *S.* Typhimurium can also be found within macrophages within the MLN (Macpherson and Uhr, 2004, Monack *et al.*, 2004a) and studies suggest that other cells or extracellular bacteria might also contribute to traffic towards this tissue (Voedisch *et al.*, 2009). Indeed, both free and cell-associated bacteria were apparently observed within pseudo-afferent lymph in mesenteric lymphadectomised rats (Tam *et al.*, 2008). Alternatively, in a model of bovine typhoid, up to 99% of *S.* Dublin found in lymph draining distal ileal loops were susceptible to gentamicin, indicating that these bacteria were extracellular (Pullinger *et al.*, 2007). However, it is possible that the proportion of cell-associated and free bacteria depends on the host and/or bacterial strain.

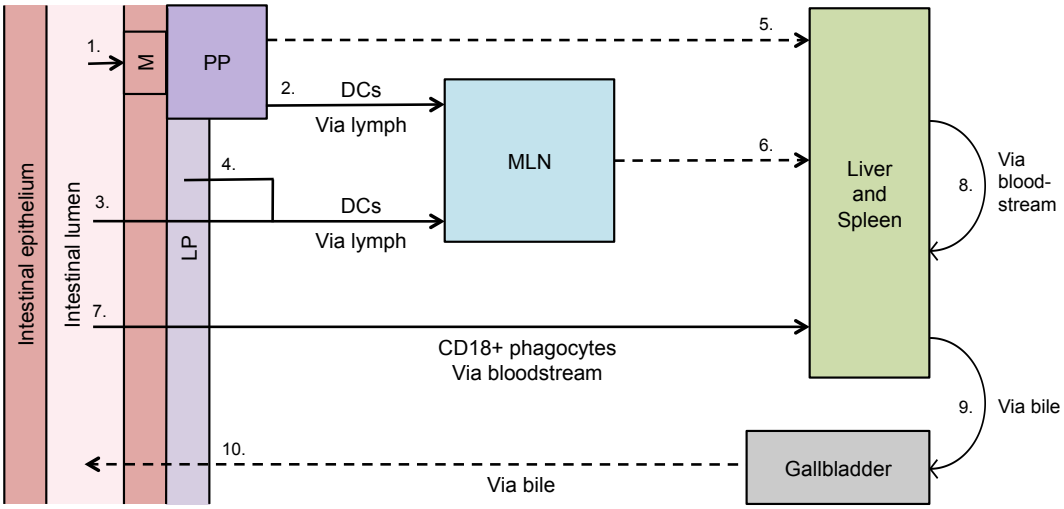


Figure 1.5. Alternative routes of dissemination of *S.* Typhimurium from the intestinal lumen to extraintestinal sites in the mouse. (1) After reaching the distal ileum, the majority of *S.* Typhimurium invade M cells that overlie PP. (2-4) Most bacteria are likely to reach the MLN by carriage within DCs via the lymphatic system. DCs transport *S.* Typhimurium from the PP (2), intestinal lumen (3) or lamina propria (LP) (4). (5) It is possible that *S.* Typhimurium can be transported directly from the PP to the liver and spleen, but large numbers of bacteria are unlikely to migrate out of the MLN (6). (7) *S.* Typhimurium can also be carried directly from the intestine to the liver and spleen via a haematogenous route involving carriage within $CD18^{+}$ phagocytes, most likely monocytes or DCs. (8) After prolonged infection, *S.* Typhimurium can be transported between organs via the bloodstream. (9) Bile excretion from the liver also carries bacteria to the gallbladder. (10) Gallbladder colonisation presumably leads to re-infection of the intestine through bile secretion.

S. Typhimurium is found in distal organs, such as the liver and spleen, after an increase in numbers in the PP and MLN (Carter and Collins, 1974). Therefore, it is thought that bacteria disseminate further to these organs after reaching the MLN. Efferent lymph from the MLN drains into the systemic circulation through the thoracic duct, therefore bacteria presumably reach the liver and spleen via the bloodstream. Indeed, after inoculation of ligated ileal loops with signature-tagged *S.* Dublin mutants in a model of bovine typhoid, the same clones in the same proportions, were found in the MLN, efferent lymph, liver and spleen (Pullinger *et al.*, 2007). However, studies of *S.* Typhimurium dissemination in murine typhoid suggest an alternative route. Mesenteric lymphadectomised mice developed exacerbated infections with *S.* Typhimurium, characterised by accelerated liver and spleen colonisation, compared to their untreated counterparts (Voedisch *et al.*, 2009, Griffin *et al.*, 2011). This indicated that the MLN might act to restrict the onward dissemination of *S.* Typhimurium. Perhaps the routes of bacterial dissemination in bovine and murine typhoid differ. However, further experiments are required to confim this, as the detailed population structure of bacteria in the MLN and spleen has not been directly compared in murine typhoid.

S. Typhimurium can also traverse the intestinal barrier and seed the spleen directly from the gut, by migrating within $CD18^+$ mononuclear phagocytes (Vazquez-Torres *et al.*, 1999) (Fig. 1.5). Further examination revealed that this involved haematogenous transport and was SPI-1 T3SS-independent. This activity has additionally been suggested to be SPI-2-dependent (Worley *et al.*, 2006). The identity of these $CD18^+$ phagocytes (DCs, monocytes or macrophages) is presently unclear. Using intravital imaging, protrusions from lamina propria DCs have been visualised taking up *S.* Typhimurium directly from the gut lumen through the intestinal epithelium (Niess *et al.*, 2005, Chieppa *et al.*, 2006). In addition, another study reported that DC-mediated traffic influenced liver and spleen colonisation by *S.* Typhimurium (Uematsu *et al.*, 2006). However, by modulating DC migration, Voedisch and colleagues found that DC-mediated traffic is not involved in transport of *S.* Typhimurium to the liver and spleen (Voedisch *et al.*, 2009). Nevertheless, since mature

macrophages are not thought to migrate out of resident tissues, it is likely that monocytes and possibly DCs represent the $CD18^+$ population that transports *S.* Typhimurium from the intestine directly to the liver and spleen. However, CD18-independent routes also account for the population of bacteria that seed the spleen, as invasion-deficient bacteria still managed to colonise the spleens of CD18-knockout mice (Vazquez-Torres *et al.*, 1999).

In conclusion, much evidence now demonstrates that *S.* Typhimurium exploits several distinct pathways and utilises a range of mechanisms to disseminate systemically from the gastrointestinal tract and colonise host tissues. However, further work is required to determine the significance of these alternative routes and the precise virulence factors involved in colonising different tissues.

1.6.2. Bacterial spreading within host tissues

Precisely how *S.* Typhimurium colonises individual organs is still not well understood. Microscopic examination of infected liver sections showed that *S.* Typhimurium-infected cells are typically found in clusters that form distinct, spatially separated foci surrounded by apparently normal tissue (Richter-Dahlfors *et al.*, 1997). Each focus most likely results from clonal expansion of one bacterial cell and spread between individual foci does not occur (Sheppard *et al.*, 2003). Interestingly, bacterial growth within organs occurs primarily as a result of an increase in the number of foci rather than the size of individual foci (Sheppard *et al.*, 2003). Therefore, some degree of bacterial dissemination from established foci to form secondary foci must also take place.

How intracellular *S.* Typhimurium spreads to new cells is a subject of debate. It is generally thought that this occurs through a process of cell death induction and subsequent phagocytosis. Indeed, *S.* Typhimurium can induce host cell cytotoxicity by various SPI-1 or 2-directed mechanisms (discussed in Sections 1.5.3.1. and 1.5.3.2.). However, the contributions of different forms of cell death to infection are poorly understood. Since the virulence of SPI-1

T3SS mutants is unaffected during the systemic phase of disease (Galan *et al.*, 1989), SPI-1-mediated cytotoxicity is unlikely to be important for organ colonisation. Instead, SPI-2-dependent apoptosis is thought to be relevant to bacterial spread, as the SPI-2 T3SS is required for growth in systemic organs (Shea *et al.*, 1999). SPI-2 dependent cytotoxicity is observed *in vitro* as a delayed form of apoptosis in macrophages and appears to involve the effectors SseL (Rytkonen *et al.*, 2007) and SpvB (Libby *et al.*, 2000). Indeed, apoptosis has been observed in *S.* Typimurium-infected livers in mice and increases throughout the infection (Richter-Dahlfors *et al.*, 1997). Exploitation of apoptotic pathways to facilitate uptake into new host cells could be beneficial for bacteria, as they would remain protected from extracellular-mediated host defences. Apoptosis is also anti-inflammatory, therefore activation of host cell microbicidal defences would be avoided. In contrast, Mastroeni and colleagues used mathematical modelling, in conjunction with microscopy data, to suggest that necrotic cell lysis, causing extracellular release of bacteria, predominates and that this occurrs stochastically (Brown *et al.*, 2006). Necrotic cell death is observed in *S. enterica* lesions in mice (Nakoneczna and Hsu, 1980, Nakoneczna and Hsu, 1983) and humans (Mert *et al.*, 2004). Therefore, it is likely that different forms of cell death contribute to spreading and organ colonisation by *S.* Typhimurium. This is further supported by the findings that both humoral and cell-mediated immune responses are required for efficient control of a *S. enterica* infection (discussed in Section 1.4).

1.6.3. Bacterial growth dynamics within systemic organs

Studying bacterial growth within infected tissues is an important aspect of understanding how *S.* Typhimurium colonises individual organs. In addition, measuring bacterial growth is a sensitive means to test the importance of bacterial and host factors in bacterial pathogenesis. Enumerating CFU in different organs of infected animals at defined time points has traditionally been used to obtain information about the global population growth dynamics of *S.* Typhimurium *in vivo*. However, to better understand the factors underlying overall growth rate, the contributions of bacterial replication and

killing must be considered, as well as the heterogeneity of growth within the bacterial population.

Early approaches to distinguish between bacterial replication and killing were based on measuring the amount and distribution of non-replicating elements within the growing bacterial population. This strategy was pioneered by Meynell, by using a superinfecting phage technique (Meynell, 1959, Maw and Meynell, 1968). Lysogenised *S.* Typhimurium were superinfected with a differentially marked mutant of their prophage that was unable to lysogenise or replicate *in vivo*. As bacteria divide, the amount of superinfecting phage is halved with each generation of replication (Fig. 1.6), while all bacteria carry the stably integrated prophage. Therefore, the mean number of divisions undergone by bacteria can be measured by determining the proportion of bacteria carrying the superinfecting phage. In this manner, up to 10 generations of bacterial replication could be measured. Furthermore, by subtracting the amount of replication measured by phage dilution from the total number of bacteria measured by CFU, the extent of bacterial killing can be determined. Maw and Meynell used phage dilution to show for the first time that the rate of replication and killing of *S.* Typhimurium in the spleens of mice was relatively low (Maw and Meynell, 1968). Later, the same technique was used to demonstrate that *S.* Typhimurium replicated more slowly in Slc11a1-resistant mice, compared to susceptible mice (Hormache 1980). Unfortunately, this technique is somewhat laborious, as the enumeration of bacteria requires induction of lysogenisation and detection of two different phages on two different indicator strains. Furthermore, stable integration and spontaneous growth of the superinfecting phage *in vivo* could produce misleading results.

A more sophisticated technique based on the principle of phage dilution was developed in 1990 (Benjamin *et al.*, 1990). Instead of a non-replicating phage, this technique involved the use of pHSG422, a plasmid with a temperature-sensitive origin of replication, that does not efficiently replicate in mice (above 30°C) (Hashimoto-Gotoh *et al.*, 1981). pHSG422 contains an antibiotic resistance marker to enable the identification of plasmid-containing bacteria.

The proportion of bacteria carrying the plasmid, following bacterial growth *in vivo*, provides a measure of the mean number of divisions undergone by the bacterial population (Fig. 1.6). Assuming plasmid-bearing and non-bearing strains are killed at equivalent rates, the amount of bacterial killing can be determined by counting the remaining number of plasmid-containing bacteria (Benjamin *et al.*, 1990). Plasmid dilution has demonstrated that Slc11a1 (Benjamin *et al.*, 1990), the *Salmonella* virulence plasmid (Gulig and Doyle, 1993) and the SPI-2 T3SS (Shea *et al.*, 1999) predominantly influence *S.* Typhimurium replication rather than killing in the mouse.

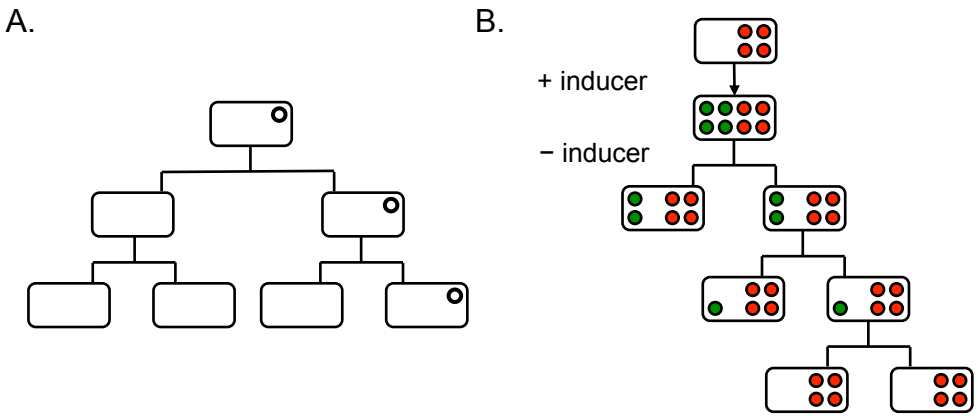


Figure 1.6. Approaches used to measure bacterial growth dynamics. (A) Principle of superinfecting phage / temperature-sensitive plasmid dilution. The number of bacteria carrying a non-replicating genetic marker halves with each division. Enumerating the proportion of bacteria carrying the marker is used to determine the number of generations of replication undergone. (B) Principle of fluorescence dilution. A pre-formed pool of inducible fluorescence (green) halves with each bacterial division. Detecting the decrease in fluorescence by flow cytometry is used to determine the number of generations of replication undergone. In this system, another fluorescent protein is expressed constitutively (red) and acts as a marker to identify all bacterial cells.

A more comprehensive analysis of global population dynamics was provided by Mastroeni and colleagues (Grant *et al.*, 2008). They used a collection of phenotypically identical wild-type *S.* Typhimurium strains that can be differentiated on the basis of different 40 base-pair (bp) DNA tags incorporated into a non-essential chromosomal site (wild-type isogenic tagged strains [WITS]). After several hours or days post-inoculation (p.i.) of mice, WITS were recovered from infected tissues and cultured on agar plates. All bacterial colonies were subsequently scraped from the plates and their

chromosomal DNA was purified. Quantifying the numbers of tagged strains by quantitative polymerase chain reaction (PCR), in conjunction with mathematical modelling, was used to generate estimates of bacterial replication and killing: increasing abundance of individual WITS and decreasing diversity of WITS provided proxy measures of mean replication and killing, respectively. Furthermore, assessing the proportion of these strains in different organs was used as an indicator of bacterial spread. In this way, bacterial growth kinetics could also be interpreted on a gross spatiotemporal level. The authors also used Phox-deficient mice, lacking the oxidative burst, to examine the influence of an innate host defence mechanism on *S.* Typhimurium growth. They found that *S.* Typhimurium undergoes rapid replication and Phox-mediated killing within hours after entering the spleen, afterwhich both replication and killing slows, as Phox-mediated resistance becomes predominantly bacteriostatic (Grant *et al.*, 2008). Interestingly, bacterial expansion was shown to be restricted to individual tissues until late stages of infection, when large numbers of bacteria accumulated within organs and haematogenous transfer of *S.* Typhimurium between different sites was observed (Grant *et al.*, 2008) (Fig. 1.5).

The approaches used to study the dynamics of bacterial population growth are becoming more sophisticated and are transforming our perception of how *S.* Typhimurium colonises host tissues. However, to gain a more detailed understanding of this, the detailed structure of bacterial populations needs to be assessed at the individual tissue level. Furthermore, observations at the tissue level must be complemented with knowledge of what occurs at the host single cell level. Therefore, our group (Helaine *et al.*, 2010) designed a technique based on fluorescence dilution (Roostalu *et al.*, 2008) to directly measure bacterial replication. This technique requires bacteria to carry a plasmid that enables the inducible production of a fluorescent protein, so that when grown in the presence of the inducer, bacteria produce this protein. Subsequent removal of the inducer prevents further protein synthesis and the preformed pool of this protein is then shared between bacteria as they divide (Fig. 1.6). Measuring the decrease in fluorescence intensity, by flow cytometry, can be used to measure bacterial replication. This is based on the

principle that the amount of fluorescent protein is halved with each bacterial division. In addition, the plasmid also encodes another fluorescent protein under the control of a constitutive promoter. This second marker allows replicating bacteria, that no longer contain detectable levels of the diluted protein, to be identified easily. In this manner, the entire population of bacteria is available for analysis. Therefore, not only could the mean replication of the bacterial population be quantified, but the variation of bacterial replication within a population could also be assessed. Furthermore, by analysing infected host cells, the replication of intracellular populations of bacteria could also be examined. In addition, similar to the method described by Meynell, coupling the direct measurement of bacterial replication by fluorescence dilution with net growth from CFU could provide a measure of bacterial killing.

The principle of dilution that forms the basis of the technique resembles carboxyfluorescein succinimidyl ester (CFSE) dye dilution that has been used extensively in immunological studies to monitor lymphocyte proliferation-dependent responses (Lyons, 2000, Wallace *et al.*, 2008). This has also been used in bacteria (Ueckert *et al.*, 1997). However, using a fluorescence marker instead of CFSE avoids the potential side effects of this ester on bacterial cell function, since CFSE covalently bonds to intracellular proteins. Fluorescence dilution has been described recently as a method to characterise differences in cell division within a bacterial population (Roostalu *et al.*, 2008). Using green fluorescent protein (GFP) dilution, Roostalu and colleagues identified a persistent population of *E. coli* in stationary-phase liquid cultures. However, they did not exploit fluorescence dilution to quantify replication rates. In addition, since their system was based only on the expression of one dilutable marker, bacteria that had replicated and emitted levels of GFP fluorescence intensity below the limit of detection were lost from analysis. The technique described in this thesis relies on the co-expression of two fluorescent markers: one for bacteria and the other for cell division. Thus, even bacteria that have gone through many rounds of division can still be identified.

In conclusion, unlike previous methods, the technique developed by our group (hereafter referred to as ‘fluorescence dilution’) should provide a direct

measure of bacterial replication at both the mean population and single bacterial cell level. This will allow the rate and diversity of replication within a population to be characterised. Furthermore, fluorescence dilution could be used to analyse the intracellular replication of bacteria within individual host cells. Therefore, applying fluorescence dilution to study *S.* Typhimurium replication in mice should provide a more comprehensive understanding of how bacterial replication contributes to colonisation of host tissues during systemic disease.

1.7. Aims of the project

The aim of my research project was to develop and validate fluorescence dilution as a tool to directly measure bacterial replication and subsequently, to use this technique to gain a more detailed understanding of the spatiotemporal replication dynamics of *S.* Typhimurium during systemic disease.

The following objectives were set:

1. To validate fluorescence dilution as a measure of bacterial replication *in vitro*.
2. To adapt fluorescence dilution as a tool to measure bacterial replication in mice.
3. To study the extent, rate and population variation in replication of *S.* Typhimurium during acute systemic disease in mice, following oral inoculation.
4. To analyse the contribution of the SPI-2 T3SS to *S.* Typhimurium replication, during a systemic infection
5. To determine the host cell type that *S.* Typhimurium preferentially replicates within, during a systemic infection.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains and plasmids

S. Typhimurium wild-type 12023 from the National Collection of Type Cultures was used in this study. Where appropriate, strains carrying the indicated plasmids (Table 2.1) were used. *E. coli* DH5〈 (Invitrogen) was used during plasmid construction.

Plasmid	Description	Source
pFPV25	promoterless *gfpmut3a*	(Cormack *et al.*, 1996)
pFPV25.1	*rpsM::gfpmut3a* promoter fusion in pFPV25	(Valdivia and Falkow, 1996)
pDsRed	P_{BAD}*::DsRed.T3_S4T* promoter fusion in pBAD18	Prof D. Bumann
pmCherry	Bacterial expression vector	Clontech
pGara	P_{BAD}*::gfpmut3a* promoter fusion in pBAD18	Mei Liu
pCara	P_{BAD}*::mCherry* promoter fusion in pBAD18	This study
pDiGc	P_{BAD}*::DsRed.T3_S4T* and *rpsM::gfpmut3a* promoter fusions in pBAD18	(Helaine *et al.*, 2010)
pDiGi	P_{BAD}*::DsRed.T3_S4T* and P_{lac}*::gfpmut3a* promoter fusions in pBAD18	(Helaine *et al.*, 2010)
pFCcGi	*rpsM::mCherry* and P_{BAD}*::gfpmut3a* promoter fusions in pFPV25	This study
pFCiGi	P_{BAD}*::mCherry* and P_{lac}*::gfpmut3a* promoter fusions in pFPV25	This study

Table 2.1. Plasmids used in this study

2.1.2. Bacterial culture conditions

S. Typhimurium was grown in Mg-MES minimal medium (MM) containing 170 mM 2-[N-morpholino]ethane-sulphonic acid at pH 5.0 or pH 2.0 where indicated, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 38 mM glycerol and 0.1% casamino acids (Beuzon *et al.*, 1999), Luria-Bertani (LB) broth, LB supplemented with 1.5% (w/v) agar or super optimal broth with catabolite repression (SOC) containing 2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5g/l NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose (Sambrook and Russell, 2001). *E. coli* DH5〈 was grown in super optimal broth (SOB) containing 2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 8.56 mM NaCl, 2.5 mM KCl (Sambrook and Russell, 2001), SOC or LB broth. Where appropriate, medium was supplemented with 0.2% (w/v) L-arabinose, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 50 µg/ml carbenicillin or 5-40 µg/ml tetracycline. Cultures were incubated at 37°C in a shaking incubator at 200 rpm with aeration unless otherwise stated. Where anaerobic conditions were required, bacteria were incubated in an anaerobic cabinet at 37°C in a reducing anaerobic atmosphere (10% CO_2, 10% H_2, 80% N_2). To ensure efficient oxygen dissipation from the media, cultures were shaken at 200 rpm for at least 1 h after placement inside the cabinet, then left standing.

2.1.3. Eukaryotic cells and culture conditions

RAW264.7 murine macrophage-like cells and HeLa human epithelial-like cells were obtained from the European Collection of Animal and Cell Cultures. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, PAA laboratories) supplemented with 10% heat inactivated fetal calf serum (FCS, PAA laboratories) at 37°C in 5% CO_2. Cells were passaged no more than 10 times.

2.1.4. Mouse strains

6-8 week or 8-12 week old female BALB/c mice (Charles River) were used where indicated. Mice were housed in a specific pathogen-free facility in filter top cages and provided food and water ad libitum.

2.1.5. Antibodies and dyes

Rabbit polyclonal anti-GFP antibody (Invitrogen) was used at a dilution of 1:500 (flow cytometry) and 1:50 000 (immunoblotting). Rabbit monoclonal anti-GFP antibody (Invitrogen) was used at a dilution of 1:50. Goat polyclonal anti-*Salmonella* common structural antigen (CSA)-1 antibody (Kirkegaard and Perry Laboratories) was used at a dilution of 1:300 (*ex vivo*) and 1:400 (*in vitro*). Alexa Fluor 488-, 633- (Invitrogen) and Cy5- (Jackson Immunoresearch Laboratories) conjugated anti-rabbit, goat and rat antibodies were used at a dilution of 1:400. Horse radish peroxidase (HRP)-conjugated goat anti-rabbit (Santa Cruz Biotechnology) antibody was used at a dilution of 1:10 000. Hamster anti-mouse CD11c- and rat anti-mouse CD11b-conjugated microbeads (Miltenyi Biotec) were used at a concentration of approximately 10 µl per 10^7 total cells. Mouse fragment, crystallisable region receptors for immunoglobulin (FcR) blocking reagent (Miltenyi Biotec) was used at a dilution of 1:10. Rat monoclonal anti-mouse F4/80 antibody (Serotec) was used at a dilution of 1:50 and the corresponding isotype control, rat anti-mouse immunoglobulin class G2b (IgG2b) antibody was used at a dilution of 1:100. DRAQ5™ (Biostatus Limited) was used at a dilution of 1:200.

2.2. Manipulation of DNA

2.2.1. Preparation of plasmid DNA

Plasmids were purified using GenElute™ Mini (Sigma-Aldrich) or QIAfilter™ Midi (Qiagen) plasmid purification kits, according to the manufacturer's instructions. DNA was eluted in sterile water. DNA purity was assessed by

electrophoresis through a 1% (w/v) agarose gel in 1 x Tris-acetate-ethylenediaminetetraacetic acid (EDTA, TAE), containing 40 mM Tris-acetate and 1 mM EDTA at pH 8.0. Plasmid yield was estimated using a NanoVue Plus (GE Healthcare).

2.2.2. PCR

Primers used in this study are listed in Table 2.2. PCR was preformed using 2.6 units of Expand High Fidelity DNA polymerase (Roche) in 50 µl, to amplify insert sequences for cloning, or 2.5 units of Taq DNA Polymerase (Sigma) in 100 µl, to check transformants. DNA polymerases were added to solution containing 1 x their respective reaction buffers, 0.2 µM of each deoxyribonucleotide triphosphate and 0.1 µM of each primer and incubated with the template DNA. Reaction conditions varied for each primer set but followed this general programme: denaturation at 95°C for 5 min; 30 cycles of denaturation at 94 - 95°C for 30 s, annealing at 50 - 60°C for 30 s and extension at 68 or 72°C for 1 to 2 min, followed by a 7 min post-well amplification at 72°C. PCR products were purified with a QIAquick® PCR purification kit (Qiagen), according to the manufacturer's instructions. Methylated template DNA was removed by digestion with *Dpn*I (NEB).

2.2.3. Construction of dual fluorescence-encoding expression vectors

When appropriate, blunting, dephosphorylation and ligations were performed with Klenow large fragment DNA polymerase (Invitrogen), shrimp alkaline phosphatase (Promega) or calf intestinal phosphatase (NEB) and T4 DNA Ligase (GE Healthcare), respectively. Digestion fragments were recovered by electrophoresis using 0.7% to 1.5% (w/v) agarose, depending on fragment size, in 1 x TAE gels and purified using a QIAquick® gel extraction kit (Qiagen), according to the manufacturer's instructions. Plasmid constructs were confirmed by assessing transformants for positive antibiotic resistance and appropriate fluorescence expression, re-digesting plasmids with appropriate enzymes and/or nucleotide sequencing.

Name	Sequence	Description
C1	5' ATC ATC **CCC GGG** AGG AGA TAT ACA TAT GGT GAG CAA GGG CGA GGA GGA TA 3'	5’ region of *mCherry* on pmCherry (forward primer); introduces the restriction site *Sma*I (bold) and a ribosomal binding site (underlined).
C2	5' AAT T**AA GCT T**TC GCG GCC GCT ACT TGT A 3'	3’ region of *mCherry* on pmCherry (reverse primer); introduces the restriction site *Hin*dIII (bold)
G1	5' GAG GAT CTG CTC ATG TTT GAC AGC TT 3'	5’ region upstream of *Cla*I on pBAD18 (forward primer)
G2	5’ ATT ATA **GCC GGC** GCT TCG TCC ATT TGA CAG GCA C 3’	3’ region downstream of *Nsi*I on pBAD18 (reverse primer); introduces the restriction site *Nae*I (bold)

Table 2.2. Primers used in this study

2.2.3.1. Construction of pFCcGi

pFPV25.1 was cleaved with *Xba*I and *Hin*dIII, to release *gfpmut3a*. *mCherry* was amplified by PCR from pmCherry using C1 and C2. The PCR product was digested and ligated into the open pFPV25.1 vector. Next, this vector was cleaved with *Eco*RV to allow insertion of a P_{BAD}*::gfpmut3a* promoter fusion. This fragment was obtained from pGFP-arabinose (pGara), which was digested with *Cla*I and *Hin*dIII.

2.2.3.2. Construction of pFCiGi

pDsRed was cleaved with *Sma*I and *Hin*dIII, to release P_{BAD}*::DsRed.T3_S4T*. The digested *mCherry* PCR product (described above) was ligated into the open pDsRed vector to generate pmCherry-arabinose (pCara). pCara was then cleaved with *Cla*I and *Hin*dIII to release the P_{BAD}*::mCherry* promoter fusion. Next, the P_{lac}*::gfpmut3a* promoter fusion was amplified by PCR from pDiGi using the primers G1 and G2 and subsequently digested with *Cla*I and *Nae*I. Finally, pFPV25 was cleaved with *Sma*I and *Hin*dIII. This open vector, along with the digested fragments containing P_{lac}*::gfpmut3a* and P_{BAD}*::mCherry* were then ligated together.

2.2.4. Nucleotide sequencing and sequence analysis

Nucleotide sequencing was carried out by the Medical Research Council Clinical Sciences Centre (Hammersmith Hospital, Imperial College London). Nucleotide sequences were analysed and translated using 4Peaks (version 1.7) and Serial Cloner (version 1.3) software.

2.2.5. Preparation of competent cells

Chemically competent *E. coli* DH5〈 were prepared by growing bacteria in 5 ml SOB medium overnight. Bacteria were next subcultured in SOB medium at a dilution of 1:100. After growth to an optical density (OD_{600}) of approximately

0.3, bacteria were kept on ice for 10 min. Next, following centrifugation at 1200 *g* for 10 min at 4°C, bacterial pellets were resuspended in cold buffer medium (10 mM potassium acetate pH 7.0, 10% glycerol, 80 mM $CaCl_2$, 20 mM $MnCl_2$, 10 mM $MgCl_2$) at a dilution of 1:0.25 the starting volume of SOB. Bacteria were kept on ice for a further 4 h and centrifuged as before, resuspending the pellet in the buffer at a dilution of 1:12 the starting volume of buffer. Bacterial suspensions were aliquoted, snap frozen in liquid nitrogen and kept at -80°C.

Electrocompetent *S.* Typhimurium were prepared by growing bacteria overnight in LB broth. Bacteria were then subcultured at a dilution of 1:200 in fresh LB broth. After growth to OD_{600} 0.4-0.6, bacteria were kept on ice and washed twice with cold water and twice with 10% cold glycerol, centrifuging at 2200 *g* for 7 min at 4°C between washes. Cells were resuspended in a final volume of 250 µl cold 10% glycerol, kept on ice and used immediately.

2.2.6. Bacterial transformations

Plasmid preparations and ligation mixtures were transformed into chemically competent *E. coli* DH5〈 cells by heat shock or electroccompetent *S.* Typhimurium by electroporation. DNA was added to 75 µl competent bacteria and incubated on ice for approximately 20 min prior to transformation. Heat shock was carried out at 42°C for 30 s followed by incubation on ice for 2 min. Electroporation was performed using an ice cold 2 mm electroporation cuvette (Molecular BioProducts) and a GenePulser II system (BioRad) at 2.5 kV, 25 mF and 200 Ω for ≥4.8 ms. Immediately after transformation, 250 µl -1 ml SOC medium was added and bacteria were allowed to recover for 1 h at 37°C at 200 rpm. Following recovery, bacteria were plated onto LB agar plates with appropriate antibiotics and incubated overnight at 37°C.

2.3. *In vitro* studies

2.3.1. Replication and fluorescence stability assays

Bacteria were grown for 9-16 h in LB broth or MM pH 5.0 supplemented with arabinose, IPTG or antibiotics, as required. Cultures were normalised to an OD_{600} of 0.01 and subcultured in fresh medium with arabinose, IPTG or antibiotics where necessary. This was defined as time = 0 h (t_{0h}). When required, arabinose or IPTG was removed by centrifugation at 16 000 *g* for 2 min or 2200 *g* for 10 min at room temperature (RT) and washing once with phosphate-buffered saline (PBS). To facilitate DsRed maturation, prior to subculture, bacteria were placed in 5 ml PBS supplemented with carbenicillin and IPTG when appropriate and incubated overnight at 37°C with aeration and shaking at 200 rpm. Where washes were performed mid-growth, pellets were resuspended in an equivalent volume of fresh MM pH 5.0 as before the wash.

Throughout the experiments, bacteria were kept at 37°C shaking at 200 rpm with aeration or in an anaerobic cabinet when indicated. Aliquots were removed at desired time-points. To quantify bacterial replication by CFU, the OD_{600} was measured and serial dilutions in PBS were plated onto duplicate LB agar plates. To assess bacterial replication by fluorescence dilution or analyse fluorescence stability, bacteria were prepared for flow cytometric analysis. To do this, bacteria were fixed by first centrifuging at 16 000 *g* for 2 min, resuspending pellets in PBS and adding paraformaldehyde (PFA) to a final concentration of 4%. Bacteria were incubated in PFA for 10 min at RT, then centrifuged as before and kept in PBS at 4°C, for no longer than 1 week, until analysis. Bacterial replication was measured as described in Section 2.9.2. Where intrabacterial GFP detection by flow cytometry or immunoblotting was required, bacteria were treated as described in Section 2.5. and Section 2.6., respectively. Where fluorescence recovery after incubation in MM pH 2.0 or anaerobic conditions was assessed, aliquots were

centrifuged at 16 000 *g* for 2 min, then resuspended in PBS and kept at 37°C shaking at 200 rpm with full aeration, until fixation and analysis by flow cytometry.

2.3.2. Infection of macrophages

All medium was warmed to 37°C before adding to cultured cells. RAW264.7 macrophages were added to wells in 24-well tissue culture plates at a density of 1 x 10^5 cells per well and incubated for 12 h to 24 h in DMEM 10% FCS before infection. Bacteria were grown for 16 h to stationary phase in MM supplemented with arabinose, IPTG or antibiotics, as required. The OD_{600} of cultures was measured and appropriate volumes of bacteria were removed (approximately 40 µl). Bacteria were then opsonised for 20 min at RT in 170 µl DMEM with 10% FCS and 10% mouse serum (Sigma). Opsonised bacteria were subsequently diluted with 600 µl DMEM with 10% FCS and 60 µl of this mixture was added to each well of macrophages at a multiplicity of infection (moi) of 10:1. This was defined as t_{0h}. To increase uptake, plates were centrifuged at 110 *g* for 5 min at RT and incubated at 37°C for a further 25 min in 5% CO_2. To synchronise infection and remove extracellular bacteria, infected macrophages were washed 2 times with PBS and incubated in DMEM 10% FCS supplemented with 100 µg/ml gentamicin for 1 h, after which they were washed again and placed in DMEM 10% FCS supplemented with 20 µg/ml gentamicin for the remainder of the infection. For sequential fluorescence dilution experiments, DMEM 10% FCS was supplemented with 12.5 µg/ml IPTG. When required, IPTG was removed as described for gentamicin.

Serial dilutions of the inoculum were plated on LB agar to confirm the moi and that similar numbers of CFU of each strain were added, as predicted by OD_{600} readings. To recover intracellular bacteria for CFU enumeration and flow cytometric analysis, cells were washed three times with PBS then lysed in 1 ml 0.1% Triton X-100 in PBS for 2-5 min at RT at indicated time points. Serial dilutions of the lysate in PBS were plated on LB agar and the remaining aliquot was centrifuged at 16 000 *g* for 2 min, to remove the detergent, and

resuspended in PBS. Bacteria were fixed as described in Section 2.3.1. and kept at 4°C until analysis. Bacterial net growth or replication was measured as described in Section 2.9.2.

2.3.3. Infection of epithelial cells

All medium was warmed to 37°C before adding to cultured cells. HeLa cells were added to wells, with or without glass coverslips when necessary, in 24-well tissue culture plates at a density of 5 x 10^4 cells per well in DMEM 10% FCS for 12 h to 24 h before infection. Bacteria were grown for 16 h in MM with arabinose and antibiotics as required, then subcultured at a dilution of 1:25 in LB broth with arabinose and antibiotics as required, until the culture reached an OD_{600} 1.5-2.0. Bacteria were then added to epithelial cells at a moi of 100:1 in Earle's Balanced Salt Solution (Invitrogen) and this was defined as t_{0h}. Invasion was allowed to proceed for 20 min at 37°C, 5% CO_2. Washes were carried out as for infection of macrophages, described in Section 2.3.2.

Serial dilutions of the inoculum were plated on LB agar to confirm the moi and that similar numbers of CFU of each strain were added, as predicted by OD_{600} readings. Invasion assays were conducted by enumerating the intracellular CFU at 2 h post-invasion, after gentamicin incubation, as described in Section 2.3.2. To examine infected cells by microscopy, coverslips were removed at desired time-points, washed three times with PBS and incubated in 4% PFA for 20 min at RT in darkness. This was followed by an additional wash in PBS and incubation in 10 mM NH_4Cl in PBS for 10 min at RT or overnight at 4°C, to remove any remaining aldehyde.

2.4. *In vivo* studies

2.4.1. Mouse infection conditions

For experiments described in Chapter 3, 8-12 week old mice were inoculated with 200 µl PBS containing a total of 1 x 10^5 or 5 x 10^5 CFU for i.p. infections and 3 x 10^8 CFU for intragastric (i.g.) infections. To prepare the inoculum,

bacteria were grown for 16 h in MM with carbenicillin, as required and subcultured at a dilution of 1:33 for 2.5 h in LB broth with carbenicillin, as required. Strains in mixed inoculums were prepared at a ratio of 1:1. Mice were sacrificed by CO_2 inhalation 48 h after i.p. inoculation or 4 and 6 days after i.g. inoculation. For experiments described in Chapters 4 and 5, 6-8 week old mice were inoculated with 200 µl of 3% $NaHCO_3$ in PBS containing 2×10^{10} CFU for i.g. infections and with 100 µl PBS containing 200-1000 CFU for intravenous (i.v.) infections. Bacteria were grown for 16 h in MM with arabinose, IPTG and antibiotics, as required, and subcultured in LB broth in the same conditions, but at a dilution of 1:10 for 3.5 h (OD_{600} 1.7). Mice were sacrificed by neck dislocation at different times post-inoculation (p.i.) and used in groups as indicated. Serial dilutions of the inoculum were plated on LB agar to confirm the correct bacterial dose was added, as predicted by OD_{600} readings.

2.4.2. Preparation of tissue samples

For experiments described in Chapter 3, spleens were removed and homogenised manually with a plastic rod, in 500 µl cold PBS. Samples were allowed to settle on ice for 5 min, after which supernatants were transferred to fresh tubes and centrifuged at 16 000 *g* for 2 min to pellet the bacteria. Cells were lysed by resuspension in 1 ml sterile water and serial dilutions in PBS were plated on LB agar for CFU enumeration. When required, strains were distinguished by fluorescence expression or growth on antibiotic-containing medium. Competitve index (CI) values were determined by dividing the ratio of wild-type and plasmid carrying strain in the output by their ratio in the input (Beuzon *et al.*, 2001).

For experiments described in Chapters 4 and 5, the small intestine (and associated PP), MLN and spleen of each mouse was recovered under aseptic conditions and pooled together. Tissues and solutions were kept on ice throughout the entire procedure. Firstly, all identifiable PP (6-10 per mouse) were removed from each small intestine and rinsed. The small intestines were then pooled together and vortexed vigorously in cold PBS to obtain the

luminal contents. The solid contents were allowed to settle twice for approximately 10 min, after which the supernatant was filtered through a 100 µm cell strainer. To lyse host cells and obtain bacterial cell suspensions, the PP, MLN and spleens were homogenised using the plastic end of a 5 ml pipette in sterile petri dishes, through the mesh of a 100 µm cell strainer in 20 ml Lysis buffer (0.3% Triton X-100 in 20% PBS). To obtain intact host cell suspensions, homogenisation was carried out as before, however 20 ml Buffer A (Ca^{2+} and Mg^{2+} free Hank's balanced salt solution [HBSS, Invitrogen], 2% FCS and 10 mM Hepes) was used instead. Following this, homogenates were passed through a 40 ⌠m cell strainer. Petri dishes and filters were rinsed once with 10 ml of the same buffer. When required, samples were centrifuged at 3-400 *g* for 5 min at 4°C, then treated with 0.83% NH_4Cl for 5 min at RT to lyse red blood cells. This reaction was stopped with the addition of 30 ml HBSS and immediate centrifugation.

2.4.3. Release of intracellular bacteria without identification of host cell type

Cell suspensions in Lysis buffer were vigorously vortexed to ensure efficient host cell lysis. These, along with the intestinal contents, were then centrifuged at 5000 *g* for 10 min at 4°C. Bacterial pellets were resuspended in PBS and either plated for CFU enumeration, on LB agar alone (MLN, spleen) or agar supplemented with carbenicillin (luminal contents, PP), or fixed in 1% PFA for 10-15 min at RT. Following fixation, samples were kept in PBS at 4°C, for no longer than 1 week, prior to flow cytometric analysis. Where intrabacterial GFP detection by flow cytometry was required, bacteria were treated as described in Section 2.5.

2.4.4. Fluorescence analysis of bacteria within specific host cell types

Unless otherwise stated, samples were centrifuged at 3-400 *g* for 5 min at 4°C. Whole cell suspensions in Buffer A were centrifuged and pellets were resuspended in Buffer B (PBS pH 7.2, 0.5% bovine serum albumin [BSA,

sigma] and 2 mM EDTA) at a concentration of approximately 100 µl per 10^7 cells. Where indicated, magnetic CD11c or CD11b-conjugated beads (Miltenyi Biotec) were then added at a concentration of approximately 10 µl per 10^7 cells, incubated at 4°C for 15 min in darkness, on a roller or standing, with gentle tapping every 5 min. To avoid non-specific CD11c labelling, samples were incubated with mouse FcR blocker (Miltenyi Biotec) at a dilution of 1:10 for 10 min at 4°C prior to incubation with CD11c-conjugated beads. Following magnetic labelling, unbound material was diluted out by addition of 1 ml (PP) or 5 ml (MLN, spleen) per 10^7 cells. Samples were centrifuged and resuspended in 50 µl (PP) or 100 µl (MLN, spleen) Buffer B per 10^7 cells. Magnetic separation was carried out according to manufacturer's instructions. This generated $CD11b^+$, $CD11c^+$ and unlabelled cell fractions (containing extracellular bacteria), where appropriate.

For fluorescence dilution analysis or CFU enumeration of intracellular bacteria, cell fractions were centrifuged and resuspended in Lysis buffer for 2 min at 4°C. Samples were then centrifuged at 16 000 *g* for 2 min, resuspended in PBS and either plated on duplicate LB agar plates or fixed in 1% PFA for 10 min at RT. Fixed samples were kept in PBS at 4°C until flow cytometric analysis. Alternatively, to examine infected cells by microscopy, approximately 1 x 10^6 cells in 40 µl Buffer B were seeded onto poly-D-lysine coated glass coverslips that had been placed into wells of a 24-well plate (BD Biocoat). Care was taken not to spill cells over the edge of the coverslips. These were allowed to adhere for 1 h at 37°C. Non-adherent cells were removed by washing once with PBS at RT and remaining cells were fixed in 1% PFA for 20 min at RT in darkness. These were then incubated in PBS with 10 mM NH_4Cl for 15 min at RT, to remove any remaining free aldehyde, then labelled with antibodies as described in Section 2.8.

2.5. Immunolabelling of *S.* Typhimurium cells in suspension

To facilitate detection of intracellular GFP, bacteria were permeabilised to allow immunolabelling of cytosolic GFP. Samples from bacterial cultures in liquid media (see Section 2.3.1.) were normalised to a concentration

representing 1 ml at an OD_{600} 0.01. Where bacteria were recovered from infected mice (see Section 2.4.3.), the entire sample was used for further analysis. Permeabilisation of bacterial cells in suspension was preformed as described in (Schlumberger *et al.*, 2005). All incubations were carried out in darkness, with rotation (for times exceeding 20 min) and in buffer volumes of no less than 100 µl. When required, samples were centrifuged at 21 000 *g* for 1 min.

To label cell-surface expressed CSA-1 and intracellular GFP in permeabilised bacteria, non-specific antibody labelling was first blocked with 10% horse serum in PBS (HS-PBS) for at least 10 min at RT. Samples were then incubated with primary antibodies for 1 h at RT. Next, samples were washed once with PBS and incubated with secondary antibodies in HS-PBS for 30 min at RT. Samples were kept in PBS at 4°C and analysed by flow cytometry within 2 h. When required, samples were centrifuged at 21 000 *g* for 1 min.

2.6. Detection of cytosolic proteins in bacterial lysates by protein immunoblotting

To ensure that equal numbers of cells were analysed, samples from bacterial cultures in liquid media were adjusted so that a volume corresponding to 1 ml of a culture of OD_{600} 0.01 was lysed in 20 µl protein sample buffer (0.25 mM Tris-Cl pH 6.8, 10% sodium dodecyl suplhate (SDS), 50% glycerol, 5% β-mercaptoethanol). Samples were boiled for 5-10 min at 100°C and used immediately or stored at -20°C. Proteins were resolved using 12% SDS-polyacrylamide gel electrophoresis (PAGE) in running buffer (25 mM Tris-Cl, 250 mM glycine, 2% SDS). SDS-PAGE gels were equilibrated in transfer buffer (44 mM Tris-Cl, 29 mM glycine, 20% methanol), for 1 min and Immunobilon-P (Millipore) membranes were activated for 10 s in methanol, washed in MilliQ water and equilibrated for 10 min in transfer buffer. The membrane and gel were placed between three sheets of filter paper soaked in transfer buffer. Transfer of proteins from the gel to the membrane was preformed using a semi-dry transfer cell (BioRad) at 80 mA for 1 h. Non-specific labelling was blocked by incubating membranes for 1 h at RT in

blocking buffer (5% milk, 0.1% Tween®-20 in PBS), on a roller. This was followed by overnight incubation at 4°C with the primary antibody diluted in blocking buffer. Membranes were washed three times for 5-10 min in 0.1% Tween-20 in PBS and incubated with the secondary antibody conjugated to HRP diluted in blocking buffer for 1 h at RT. Five further washes were carried out as before, after which proteins were detected using the enhanced chemiluminescence detection system (GE Healthcare) according to manufacturer's instructions and high performance chemiluminescence film (GE Healthcare).

2.7. Flow cytometry

All samples recovered from infected murine tissues were filtered immediately prior to analysis using a 40 µm cell strainer. Wild-type *S.* Typhimurium or tissues from uninfected mice were used to control for autofluorescence. Secondary antibodies alone were used to control for non-specific antibody labelling. For each sample 10 000 - 30 000 events (liquid culture), 50 000 events (macrophage infection) or up to 10 000 events (mouse infections) were analysed. Samples were acquired on a FACSCalibur cytometer (BD) using Cell Quest Pro software or on a LSR II (BD) or LSRFortessa cytometer (BD) using FACSDiva software. Fluorophores were excited at 488 nm, 532 nm or 561 nm and 640 nm, when appropriate. On the FACScalibur, green and red fluorescence were detected at 510/20 nm and 595/20 nm, respectively. On the LSR II and LSRFortessa, green fluorescence was detected at 525/50 or 530/30 nm, red fluorescence at 610/10 or 610/20 nm and far-red fluorescence at 670/14 nm. Data were analysed with FlowJo Software version 8.6.6. (Tree Star, Inc.). For single red and green fluorescence dilution, bacteria were gated on high green or red fluorescence, respectively. When both fluorescent proteins were diluted *in vivo*, bacteria were identified based on anti-CSA-1 labelling and fluorescence expression as described in Section 4.2.5.1. Bacterial fluorescence is represented in dot plots or as histograms that show the frequency of events at each intensity of fluorescence obtained.

2.8. Fluorescence microscopy

When required, coverslips were washed once in PBS and incubated with primary antibodies in HS-PBS for 1 h at RT in darkness in a humidified chamber. Coverslips were then washed twice with PBS and incubated with secondary antibodies in HS-PBS for 30 min as before. Following another two washes with PBS, cell nuclei were stained with DRAQ5™ in PBS containing 0.5 mg/ml Ribonuclease A (Sigma) for 20 min as before. Lastly, labelled (or unlabelled) coverslips were washed twice with PBS and distilled water, mounted onto glass slides using Mowiol and kept at 4°C for at least 12 h until observation. All cells were examined on an epifluorescent microscope (Zeiss AxioImager.D1) using a 100x oil immersion objective and this was also used for differential interference contrast (DIC) imaging. Z-stacks were acquired on a confocal laser scanning microscope (Zeiss Axiovert LSM510). Images were processed using Adobe Photoshop software (Adobe Systems).

2.9. Data analysis

2.9.1. Processing of *in vivo* fluorescence dilution data

Since very few events might not provide a profile representative of the entire population, bacterial fluorescence data was not quantified in experiments where only a total of 20 or less bacteria were detected in a sample. Bacterial populations recovered from the small intestinal lumen (SI) and PP, which displayed fluorescence profiles that were characteristic of replication-independent GFP fluorescence loss (heterogeneous, low intensity of green fluorescence), were excluded from further analysis. The proportions of replicating and non-replicating bacteria were subsequently calculated with respect to the remaining population(s) of bacteria. When no replication-independent GFP fluorescence loss was observed, the proportions of replicating and non-replicating bacteria were calculated with respect to the total population of bacteria detected. The fluorescence of replicating bacteria was not quantified if the proportion of these bacteria represented less than 10% of the total population and at the same time, represented only 20

bacteria or less. This was because so few events might not be representative. Since fluorescence dilution cannot accurately measure more than 6 generations of replication, measurements of approximately 6 generations or more were not examined further, as these were likely to represent an underestimation of the true extent of division.

2.9.2. Measurement of bacterial replication and killing

Bacterial replication profiles measured by CFU and fluorescence dilution were generally represented by the increase in generations over time. Fold replication (*F*) for CFU (1) and fluorescence dilution (2) were calculated using the following equations:

$$(1) \qquad F_t = \frac{C_{(t)}}{C_0} \qquad \text{or} \qquad (2) \qquad F_t = \frac{C_0}{C_{(t)}}$$

where F_t is fold at time (t), C_t is the cell count (CFU/ml) or the geometric mean of fluorescence intensity (MFI) at time (t) and C_0 is the cell count or MFI at t = 0. Fold replication and generation number (N) are related as follows:

$$F = 2^N$$

This is derived from the principle that cell counts double with each division or generation, while the fluorescence intensity is halved. Therefore, the number of generations measured by cell counts and fluorescence dilution at a specified time (N_t) were calculated as:

$$N_t = \frac{\ln (F_t)}{\ln (2)}$$

To generate a killing rate or index (generations/h), the rate of net growth (generations/h) obtained by CFU was subtracted from the replication rate (generations/h) obtained by fluorescence dilution (Helaine *et al.*, 2010). Where indicated, the rate of replication was also calculated as h/generation.

2.10. Statistical analysis

Data were analysed using a Student's *t* test. The parameters used in each analysis are indicated as appropriate. Significance was indicated by a probability (*p*) value < 0.05. Unless otherwise indicated, graphs show the mean ± one standard error from the mean (SEM) for a least 3 independent experiments.

3. VALIDATION OF FLUORESCENCE DILUTION AS A TOOL TO MEASURE BACTERIAL REPLICATION *IN VITRO* AND ADAPTATION OF THE TECHNIQUE FOR USE *IN VIVO*

3. 1. Introduction

To provide a direct measure of intracellular bacterial replication, our group (Helaine *et al*., 2010) designed a technique based on fluorescence dilution. This system uses the decrease in fluorescence intensity resulting from halving of a pool of fluorescent protein within a bacterial cell as a surrogate marker for cell division (Fig. 1.6B). In this chapter, I describe the validation of fluorescence dilution as a tool to measure *S.* Typhimurium replication in different environments *in vitro*. I also describe the development of optimised systems to allow fluorescence dilution to be applied to murine studies, so that this technique can be used to characterise *S.* Typhimurium replication during murine typhoid disease.

3.2. Results

3.2.1. Measurement of *S.* Typhimurium replication *in vitro* by fluorescence dilution

3.2.1.1. pDiGc fluorescence reporter system

Our fluorescence dilution method requires bacteria to carry a dual fluorescence-encoding reporter plasmid (Fig. 3.1A). This was constructed and transformed into *S.* Typhimurium 12023. The vector backbone was derived from the bacterial expression vector pBAD18 and has a ColE1 origin of replication (ori) (Guzman *et al.*, 1995). In this vector, the *repressor of primer* (*rop*) gene is disrupted, giving rise to a high plasmid copy number of approximately 50-100 copies per cell (Twigg and Sherratt, 1980, Cesareni *et al.*, 1982). This plasmid encodes a *Discosoma*-red fluorescent protein variant DsRed.T3_S4T (DsRed) that has been optimised for faster maturation (Bevis and Glick, 2002) and brightness (Sorensen *et al.*, 2003) in bacteria. DsRed was placed under the regulation of the arabinose-inducible promoter P_{BAD}. In

addition, enhanced GFP, encoded by *gfpmut3a* (Cormack *et al.*, 1996), was placed under the regulation of the constitutive promoter *rpsM* (Valdivia and Falkow, 1996). DsRed and GFP have been widely used in *S. enterica* research and their spectral properties allow them to be easily distinguished when expressed simultaneously (Table 3.1). A gene for ampicillin resistance is present in the plasmid to ensure plasmid maintenance. Hereafter, this plasmid will be referred to as pDiGc (DsRed-inducible GFP-constitutive).

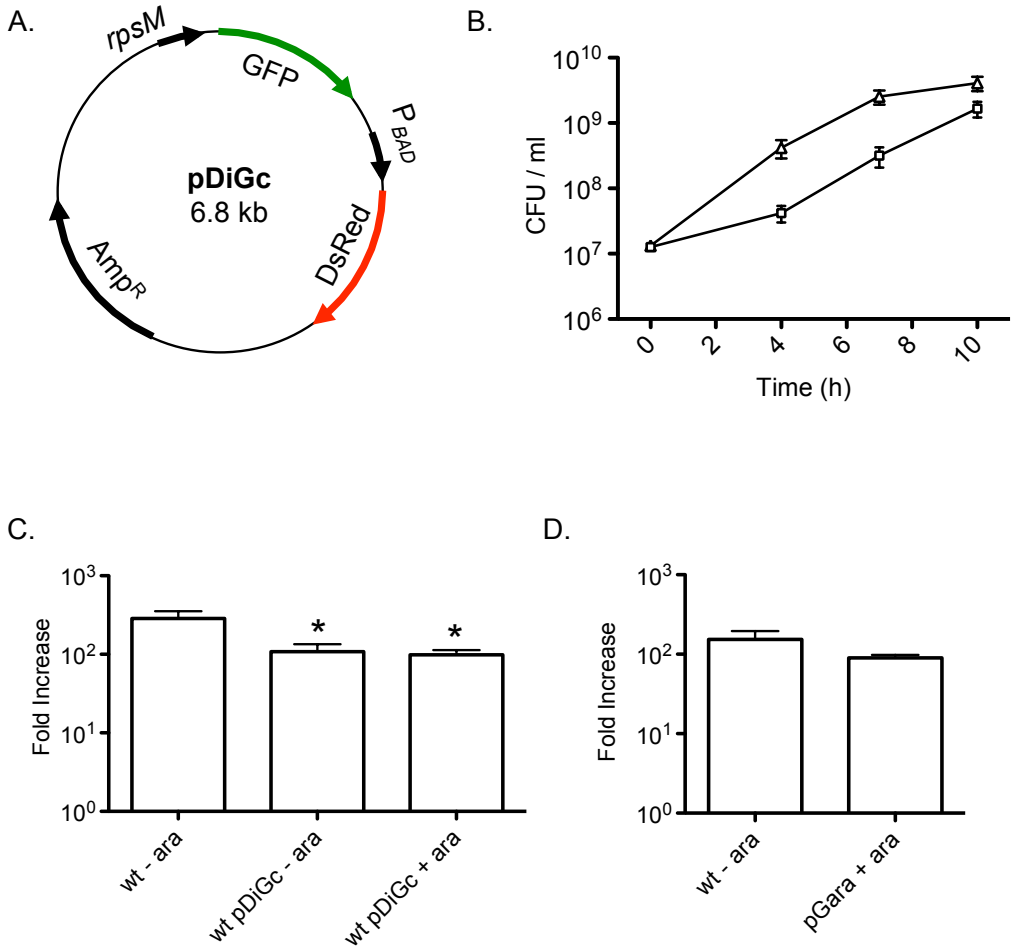


Figure 3.1. Influence of pDiGc on *S.* Typhimurium replication *in vitro*. (A) Map of pDiGc. DsRed and GFP are under the control of the arabinose-inducible P_{BAD} promoter and the constitutive *rpsM* promoter, respectively. Amp^R denotes the gene for ampicillin resistance. The vector backbone is derived from pBAD18. (B) Net growth of wild-type *S.* Typhimurium (triangles) and the same strain carrying pDiGc (squares) in MM without arabinose, determined by CFU enumeration. (C-D) Wild-type *S.* Typhimurium and the same strain carrying pDiGc (C) or pGara (D) were grown overnight in MM, with or without arabinose where indicated. Net growth in RAW macrophages from 2 h to 16 h post-uptake was determined by fold change in CFU enumeration at these time points. Data represent the mean ± SEM for 2 (D) or 3 (A-C) independent experiments. Data were analysed using a paired, one-tailed Student's *t* test and values that were significantly different ($P < 0.05$) from the wild-type strain carrying no plasmid are indicated with an asterisk (*).

Protein	Origin	Organism	Excitation (nm)	Emission (nm)	$t_{0.5}$ for maturation at 37°C	pKa	*in vivo* structure
Enhanced GFP	GFP	*Aequorea victoria*	488	507	≤ 8 min	6	weak dimer
DsRed.T3_S4T	DsRed	*Discosoma striata*	560	589	ND (2.5 h)*	ND (4.7)**	tetramer
mCherry	DsRed	*Discosoma striata*	587	610	15 min	<4.5	monomer

Table 3.1. Fluorescent protein properties
Properties of GFP, DsRed and mCherry. Adapted from Shaner *et al.,* (2005), Shaner *et al.,* (2004), Bevis and Glick (2002), Cormack *et al*., (1996). (*) indicates data for DsRed.T3 and (**) indicates data for wild-type DsRed.

3.2.1.2. Influence of pDiGc carriage and associated fluorescent protein production on *S.* Typhimurium replication in minimal medium and macrophages

The growth of wild-type *S.* Typhimurium and the same strain carrying pDiGc was compared during culture in MM at pH 5.0, as this medium is thought to mimic the conditions experienced by *S*. Typhimurium inside the SCV in macrophages (Beuzon *et al.*, 1999). Experiments were conducted in the absence of arabinose and growth was measured by enumerating bacterial CFU. *S.* Typhimurium carrying pDiGc grew more slowly than the wild-type strain (Fig. 3.1B). However, bacterial counts of both strains were similar by 10 h.

Given that the growth of *S.* Typhimurium carrying pDiGc was reduced in MM and that some expression vectors and the production of certain fluorescent proteins have been implicated in reducing bacterial virulence (Coulson *et al.*, 1994, Wendland and Bumann, 2002, Rang *et al.*, 2003, Abromaitis *et al.*, 2005, Clark *et al.*, 2009, Knodler *et al.*, 2005a), I examined the influence of pDiGc on *S.* Typhimurium growth during infection of the RAW264.7 macrophage-like cell line. RAW264.7 macrophages were infected with wild-type *S.* Typhimurium and the same strain carrying pDiGc that had been grown overnight in MM with or without arabinose (as indicated). The infection was allowed to proceed in the absence of arabinose and the fold increase in bacterial CFU between 2 h and 16 h was measured. The fold increase of *S.* Typhimurium carrying pDiGc was over 2.5 times less than the wild-type strain ($p = 0.0144$ and 0.0183 for strains grown without and with arabinose, respectively) (Fig. 3.1C). However, there was no significant difference in growth between *S.* Typhimurium carrying pDiGc, when grown in the presence or absence of arabinose, showing that the production of DsRed did not impair bacterial growth (Fig. 3.1C). Furthermore, arabinose-induced bacteria carrying pGara, which is another derivative of pBAD18 that instead encodes GFP downstream of P_{BAD}, or bacteria carrying pFPV25.1, from which GFP is synthesised constitutively, grew at a similar rate as the wild-type strain in RAW264.7 macrophages (Fig. 3.1D and data not shown). This indicates that

the production of GFP also did not impair bacterial growth, which is consistent with other reports (Knodler *et al.*, 2005a, Clark *et al.*, 2009). Therefore, the attenuated growth of *S.* Typhimurium carrying pDiGc in macrophages is due to the burden or nature of the plasmid DNA, rather than fluorescent protein production *per se*.

3.2.1.3. Validation of fluorescence dilution using pDiGc

To test the regulation of fluorescent protein production in *S.* Typhimurium carrying pDiGc, bacteria were grown overnight in MM containing arabinose and then subcultured in MM without or with arabinose. Bacteria were fixed and their fluorescence was detected by flow cytometry (Fig. 3.2A; adapted from BSc report). In the presence of arabinose, both red and green fluorescence were expressed at high levels (Fig 3.2A). Removing arabinose resulted in the loss of red fluorescence with bacterial growth (Fig. 3.2B). As expected, this loss was not observed when arabinose was maintained (Fig. 3.2C). Bacteria were also subcultured for 24 h in MM containing a bacteriostatic concentration of tetracycline, an antibiotic known to inhibit bacterial protein synthesis by interfering with ribosomal function (Goldman *et al.*, 1983). This confirmed that in the absence of bacterial replication and protein synthesis, high red and green fluorescence levels were maintained (Fig. 3.2D). This demonstrates that DsRed and GFP are stable over at least 24 h.

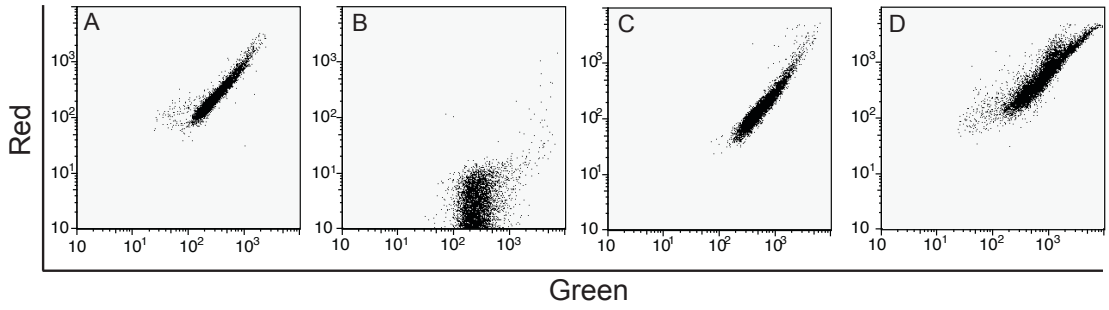


Figure 3.2. Validation of fluorescence dilution using pDiGc. Flow cytometric detection of red and green fluorescence expression by *S.* Typhimurium carrying pDiGc after growth overnight in MM with arabinose (A) and subsequent subculture for 24 h in MM without arabinose (B) with arabinose (C) and with a bacteriostatic concentration of tetracycline (D).

3.2.1.4. Bacterial replication measured by single (DsRed) fluorescence dilution

Next, I determined the suitability of fluorescence dilution to measure bacterial replication. *S.* Typhimurium carrying pDiGc was grown for 9 h in MM containing arabinose, so that all bacteria expressed both red and green fluorescence. This was followed by an overnight incubation at 37°C in PBS without arabinose, to facilitate maximum production of red fluorescence through maturation of DsRed in the absence of bacterial growth. Following incubation in PBS, bacteria were subcultured in MM without arabinose and growth was monitored by fluorescence dilution. Removing arabinose resulted in the dilution of red fluorescence with bacterial growth (Fig. 3.3A). This dilution proceeded in a homogenous manner, represented by a series of overlapping, normally distributed curves, which is indicative of uniform growth by the entire bacterial population.

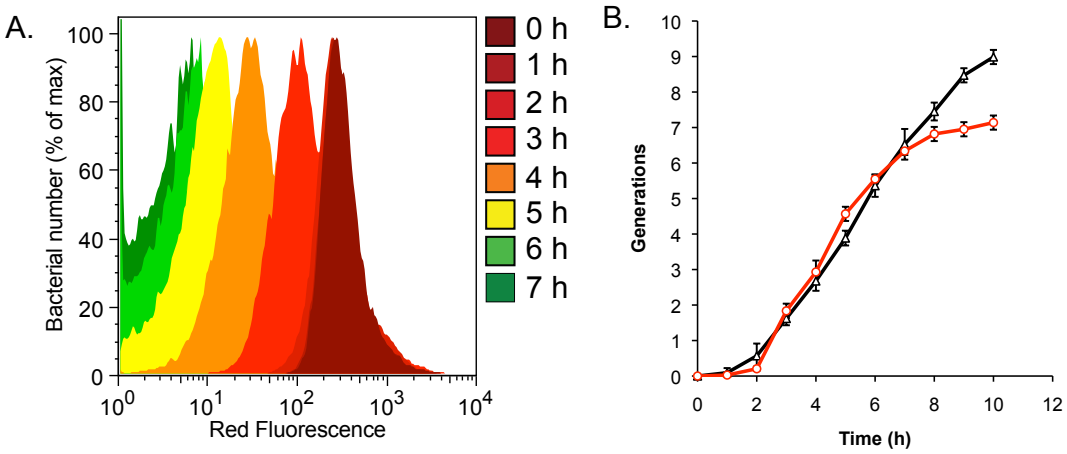


Figure 3.3. Bacterial replication measured by single (DsRed) fluorescence dilution. Pre-induced *S.* Typhimurium carrying pDiGc subcultured in MM without arabinose. (A) Representative red fluorescence profiles detected by flow cytometry at hourly time-points. (B) Bacterial replication measured by CFU (black) and DsRed dilution (red). Data represent the mean ± SEM for at least 3 independent experiments.

Enumeration of CFU is the most widely used method to measure bacterial net growth. Accordingly, I compared the replication profile determined by fluorescence dilution during growth in MM with that obtained by measuring bacterial CFU. The replication profiles obtained by both methods were indistinguishable for more than 6 generations (Fig. 3.3B) and indicated

replication at a rate of approximately 1 h per division. Thereafter, CFU counts indicated continued bacterial growth, whereas a plateau was observed in the replication profile determined by fluorescence dilution (Fig. 3.3B). This divergence represents the dilution of red fluorescence intensity to levels below the limit of detection. Therefore, these data demonstrate that fluorescence dilution provides an alternative to CFU to monitor bacterial replication, over at least 6 generations.

3.2.2. Sequential fluorescence dilution *in vitro*

3.2.2.1. pDiGi reporter system

I hypothesised that the dynamic range of fluorescence dilution could be extended by initiating the dilution of a second fluorescent protein (GFP), following the dilution of DsRed. To enable the sequential dilution of DsRed and GFP, a modified version of pDiGc was constructed. The constitutive promoter *rpsM* was replaced by the IPTG-inducible promoter P_{lac}, so that both GFP and DsRed were under inducible control (Fig. 3.4A). Hereafter this plasmid will be referred to as pDiGi (DsRed-inducible GFP-inducible).

3.2.2.2. Influence of pDiGi carriage on *S.* Typhimurium replication in minimal medium

I examined the fitness cost of pDiGi carriage on *S.* Typhimurium by comparing the growth of wild-type bacteria and the same strain carrying pDiGi. Experiments were conducted in MM without arabinose and growth was measured by CFU enumeration. *S.* Typhimurium carrying pDiGi grew slightly slower than the wild-type strain (Fig. 3.4B). However, this reduction was not as marked as in the strain carrying pDiGc (Fig. 3.1B) and bacterial counts of both strains were similar by 8 h (Fig. 3.4B). Therefore, pDiGi does not exert as great a fitness cost on *S.* Typhimurium as pDiGc.

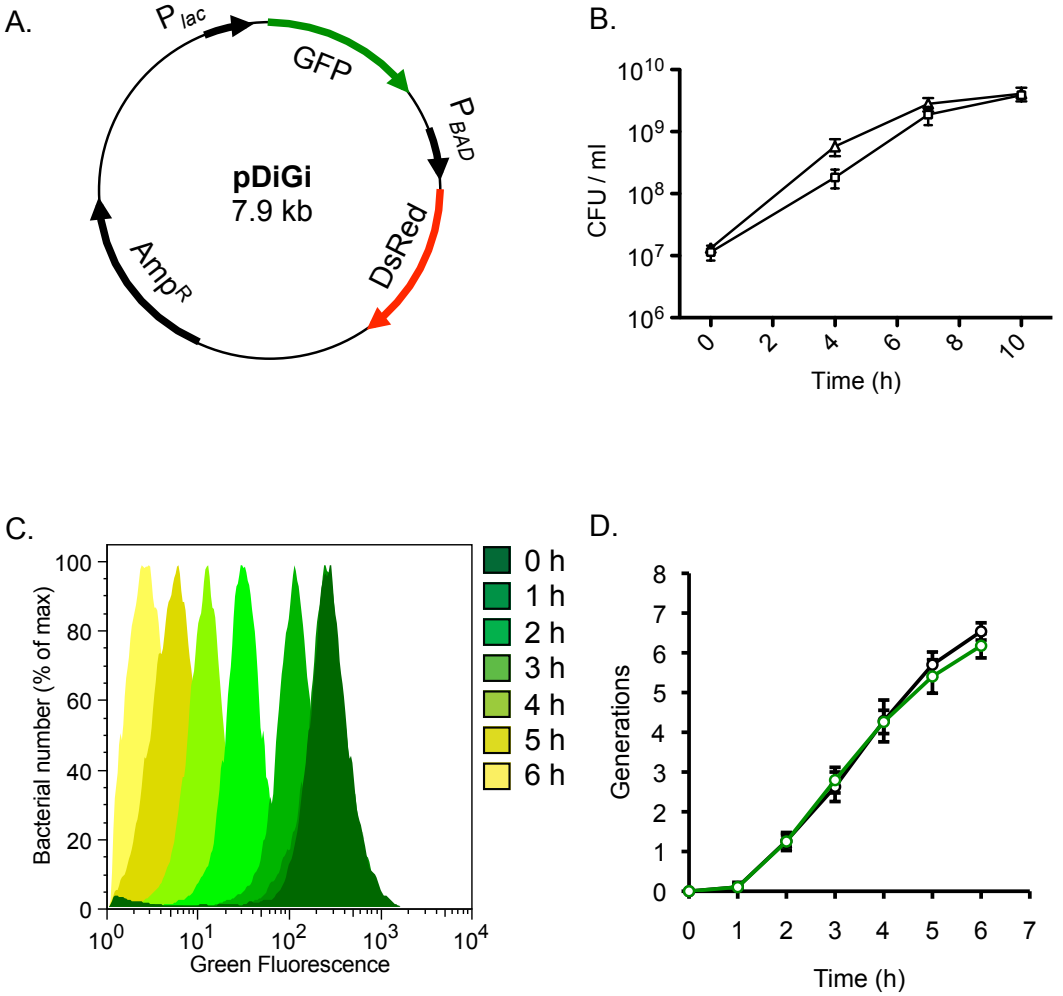


Figure 3.4. Bacterial replication measured by single (GFP) fluorescence dilution. (A) Map of pDiGi. DsRed and GFP are under the control of the arabinose-inducible P_{BAD} and the IPTG-inducible P_{lac} promoters, respectively. Amp^R denotes the gene for ampicillin resistance. The vector backbone is derived from pBAD18. (B) Net growth of wild-type *S.* Typhimurium (triangles) and the same strain carrying pDiGi (squares) in MM without arabinose, determined by CFU enumeration at designated time-points. (C-D) Pre-induced *S.* Typhimurium carrying pDiGi subcultured in MM without arabinose or IPTG. (C) Representative profiles of bacterial green fluorescence detected by flow cytometry at hourly time-points. (D) Bacterial replication measured by CFU (black) and GFP dilution (green). Data in B and D represent the mean ± SEM for 3 independent experiments.

3.2.2.3. Bacterial replication measured by single (GFP) fluorescence dilution

Prior to monitoring the sequential dilution of DsRed and GFP, it was important to establish whether GFP dilution was also suitable to measure bacterial replication. *S.* Typhimurium carrying pDiGi was grown overnight in MM containing IPTG, to induce GFP production. Bacteria were then subcultured in

MM without IPTG. Removal of IPTG resulted in the dilution of green fluorescence with bacterial growth (Fig. 3.4C) and these profiles resembled those obtained by DsRed dilution (Fig. 3.3A). As expected, comparison of the replication profiles obtained by both CFU and fluorescence dilution were indistinguishable over more than 6 generations (Fig. 3.4D). Thereafter, the two profiles diverged as described earlier (Fig. 3.3B), as a result of a limitation in the sensitivity of detection of low levels of fluorescence intensity. This confirms that monitoring the dilution of GFP is also a suitable means to measure bacterial replication, for over at least 6 generations.

3.2.2.4. Bacterial replication measured by sequential (DsRed and GFP) fluorescence dilution

Having confirmed that DsRed and GFP dilution were both suitable measures of bacterial replication and could independently monitor over 6 generations of replication, I next investigated the extent of replication that could be determined by the sequential dilution of these two fluorescent proteins.

S. Typhimurium carrying pDiGi was grown in MM containing arabinose and IPTG, to induce red and green fluorescence production, respectively. After full DsRed maturation in PBS, bacteria were subcultured in MM with IPTG but without arabinose, to initiate DsRed dilution (Fig. 3.5A). After 4 h, before the limit of DsRed fluorescence dilution was reached (Fig. 3.5C), IPTG was removed, to initiate GFP dilution (Fig. 3.5B). Samples were removed at hourly intervals and replication was monitored by CFU and bacterial fluorescence dilution (Fig. 3.5C). The removal of IPTG did not affect bacterial replication (Fig. 3.5D). After removal of IPTG, bacteria were allowed to grow for a further 4 h, by which time they had reached stationary phase. Comparison of the replication profiles obtained by fluorescence dilution and CFU shows that at least 10 generations of bacterial replication (corresponding to a greater than 1000-fold increase in bacterial numbers) can be measured by sequential fluorescence dilution (Fig. 3.5C). Bacterial growth was restimulated by carrying out a further subculture without arabinose and IPTG at 8 h. This confirmed that the limit of GFP fluorescence detection had been reached after

10 generations of bacterial replication, as no further increase in bacterial replication was measurable by GFP dilution, despite the increase in CFU (Fig. 3.5C).

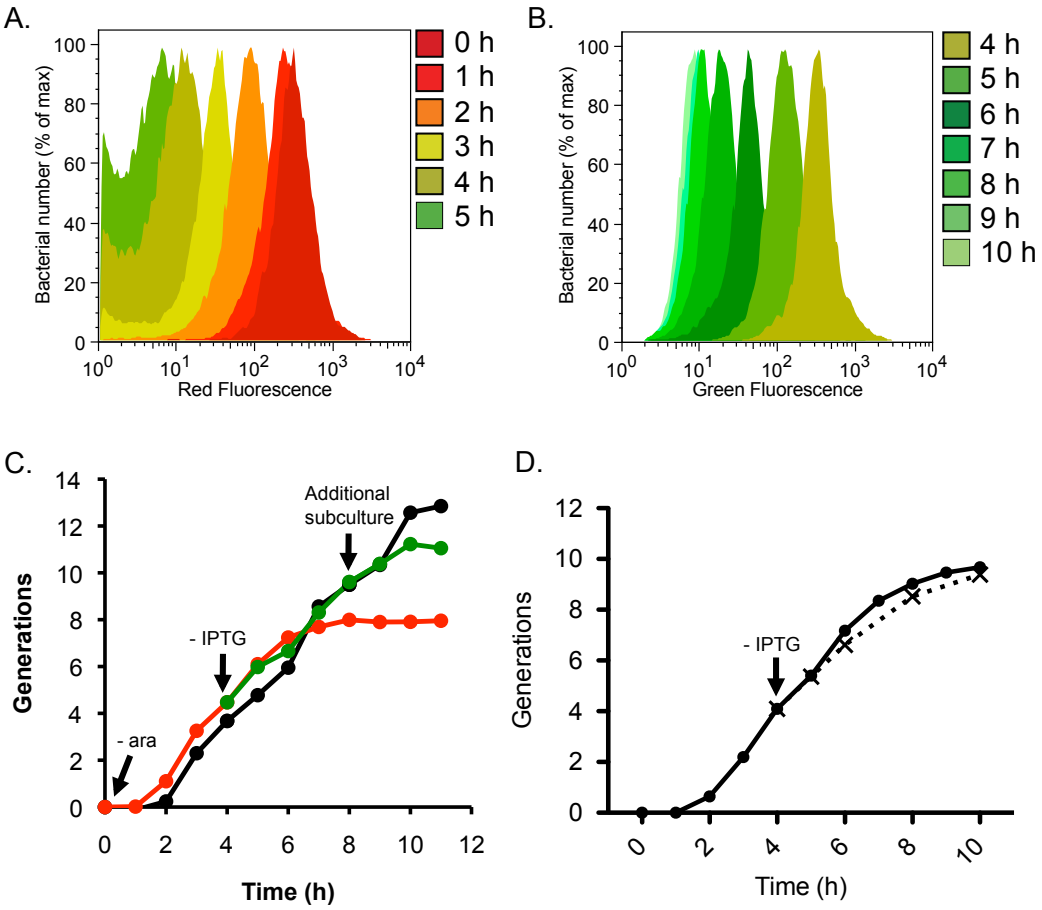


Figure 3.5. Bacterial replication measured by sequential fluorescence dilution. (A-B) Flow cytometric detection of red (A) and green (B) fluorescence from pre-induced *S.* Typhimurium carrying pDiGi after removal of arabinose and IPTG, respectively. (C) Bacterial replication measured by CFU (black) and sequential dilution of DsRed (red) and GFP (green). Arrows indicate removal of arabinose at t_{0h}, IPTG at t_{4h} and initiation of an additional subculture at t_{8h}. Data are representative of 3 independent experiments. (D) Growth of pre-induced *S.* Typhimurium carrying pDiGi during subculture in MM with IPTG and without arabinose (solid line). Growth was determined by CFU enumeration at hourly intervals. IPTG was washed out from half the culture at t_{4h} (arrow) and growth continued to be measured by CFU every 2 hours, in the absence of IPTG (dotted line). Data are representative of 3 independent experiments.

3.2.3. Measurement of *S.* Typhimurium replication in macrophages

3.2.3.1. Intramacrophage replication measured by single fluorescence dilution

Having confirmed that fluorescence dilution provides a suitable measure of bacterial replication, I used this technique to measure the replication of *S.* Typhimurium in macrophages. RAW264.7 macrophages were infected with *S.* Typhimurium carrying pDiGc that was grown overnight in MM containing arabinose, to induce DsRed production. A DsRed maturation step in PBS was not required in these experiments. This was because preliminary results showed that full DsRed maturation occurred by 2 h post-uptake into macrophages, as *S.* Typhimurium does not replicate significantly during this time period. The infection was conducted in the absence of arabinose and bacterial fluorescence was monitored by flow cytometry every 2 h. Bacteria replicated at a rate of approximately 4 h per division from 2 h to 6 h (Fig. 3.6A). Thereafter, the replication increased to a rate of approximately 1 h per division, which was comparable to the replication rate in MM. After 10 h, no further dilution of DsRed could be detected. This plateau could represent either a true reduction in the rate of bacterial replication or the limit of fluorescence detection. Intramacrophage replication was subsequently measured by sequential fluorescence dilution, to discriminate between these two possibilities.

3.2.3.2. Measurement of *S.* Typhimurium replication in macrophages by sequential fluorescence dilution

RAW264.7 macrophages were infected with *S.* Typhimurium carrying pDiGi that was grown overnight in MM containing arabinose and IPTG, to induce both red and green fluorescence production, respectively. The infection was conducted in the absence of arabinose, however IPTG was maintained until 8 h post-uptake. Replication was determined by monitoring changes in bacterial fluorescence intensity every 2 h. Bacterial replication measured by DsRed dilution displayed similar kinetics to the experiment described above, using

pDiGc (Fig. 3.6B). On removal of IPTG at 8 h post-uptake, GFP dilution proceeded in a manner representative of continued rapid bacterial replication (approximately 1.5 h per division) and at 16 h post-uptake bacteria appeared to have replicated over approximately 400 fold. This indicated that the previous plateau observed by DsRed fluorescence dilution (Fig. 3.6A) was indeed due to a limitation in detection of low levels of fluorescence intensity.

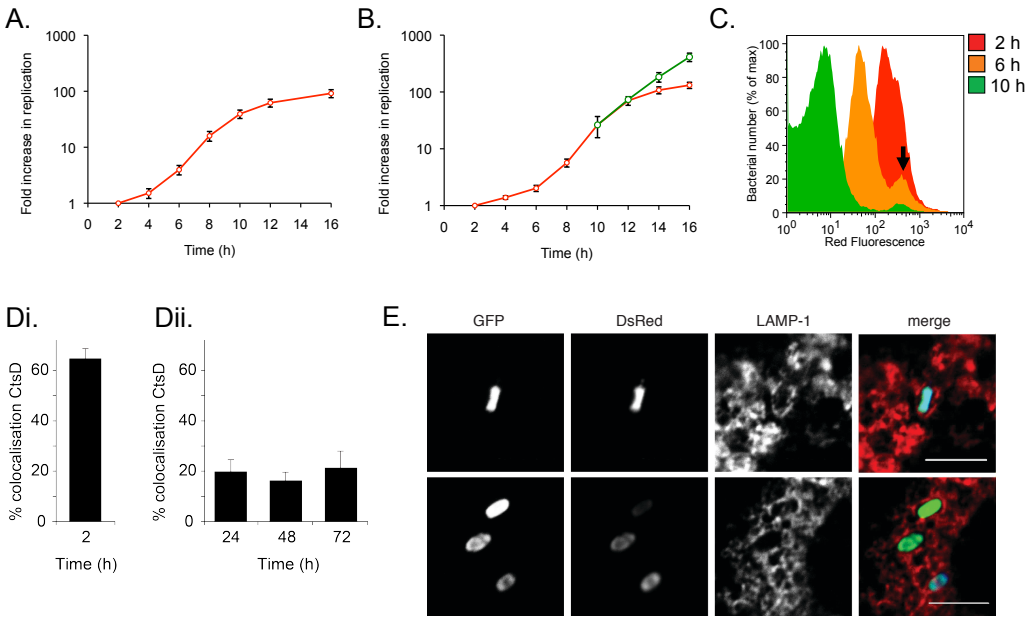


Figure 3.6. Characterisation of *S.* Typhimurium replication in macrophages by fluorescence dilution. (A) Replication of pre-induced *S.* Typhimurium carrying pDiGc in RAW macrophages after removal of arabinose. At 2 h intervals, bacterial replication was measured DsRed dilution. (B) Replication of *S.* Typhimurium carrying pDiGi in RAW macrophages. At 2 h intervals, replication was measured DsRed (red) and GFP (green) dilution, after removal of arabinose and IPTG, respectively. (C) Representative red fluorescence profiles, detected by flow cytometry, of *S.* Typhimurium carrying pDiGc, at indicated timepoints post-uptake, after removal of arabinose. Arrow indicates non-replicating bacteria. (D) Proportion of heat killed (i) and non-replicating (ii) *S.* Typhimurium that colocalise with Cathepsin D (CtsD) at designated times post-uptake by bone marrow derived (bm) macrophages (n = 150–300 macrophages). Quantification was carried out at different times, as heat-killed bacteria are no longer visible after 2 h p.i. and non-replicating bacteria can only be adequately distinguished from replicating bacteria after 24 h p.i. (E) Fluorescence microscopy of bm macrophages infected with *S.* Typhimurium carrying pDiGc at 24 h post-uptake, showing replicating bacteria (green only) and those that have not replicated (green and red), surrounded by LAMP-1 positive vacuoles. Scale bars: 10 μm. Unless otherwise indicated, data represent the mean ± SEM for at least 3 independent experiments. Data in (D) and (E) are provided by Dr. J. Thompson.

Interestingly, unlike growth in MM, the fluorescence profiles of *S.* Typhimurium inside macrophages revealed that the bacterial population was heterogeneous (Fig. 3.6C). A sub-population of bacteria replicated in a uniform manner, while another sub-population did not replicate. Dr J. Thompson of our group conducted experiments to further examine the intracellular sub-population of *S.* Typhimurium that persisted without

replicating. This demonstrated that the majority of heat-killed bacteria colocalised with the lysosomal enzyme Cathepsin D (Fig. 3.6 Di), whereas non-replicating bacteria did not (Fig 3.6Dii), persisting instead within a lysosomal-associated membrane protein (LAMP)-1^+ vacuole (Fig. 3.6E), characteristics of normal SCVs (Helaine *et al*., 2010).

3.2.4. Adaptation of fluorescence dilution for murine studies

3.2.4.1. Suitability of pDiGc for murine studies

The main aim of this study was to apply fluorescence dilution to characterise the replication dynamics of *S.* Typhimurium during murine typhoid. Therefore, after validating the technique *in vitro* and using the pDiGc reporter system to enable measurement of *S.* Typhimurium replication in MM and macrophages by fluorescence dilution, I assessed the behaviour of the pDiGc reporter system *in vivo*. Experiments were conducted to determine the impact of pDiGc on *S.* Typhimurium virulence in mice and to assess the suitability of this strain as a model to study the replication dynamics of *S.* Typhimurium following i.g. inoculation of mice.

pDiGc was stably maintained in *S.* Typhimurium after 2 days in mice (Table 3.2). However, *S.* Typhimurium carrying pDiGc displayed a significant virulence attenuation with respect to the wild-type strain (CI = 0.003) after i.p. inoculation and also displayed low levels of spleen colonisation after 2 days post-i.p. inoculation (Dr. S. Helaine, personal communication). Moreover, preliminary invasion assays conducted using HeLa epithelial cells revealed that *S.* Typhimurium carrying pDiGc were unable to invade non-phagocytic cells, which is required for efficient penetration of the intestinal wall and establishment of a systemic infection in orally inoculated mice (Galan *et al.*, 1989). It is possible that carriage of pDiGc may be associated with suppressed *prgH* activity (Clark *et al.*, 2009), which would indicate low levels of SPI-1 T3SS induction. However, this was not investigated. Collectively, these results demonstrate that, although pDiGc is a useful reporter system for

studying bacterial replication in macrophages *in vitro*, this plasmid is unsuitable for *in vivo* studies.

	Total number of bacteria (CFU / spleen)	Number of bacteria carrying pDiGc (CFU / spleen)	pDiGc stability (%)
1	2.5×10^6	2.5×10^6	100
2	1.4×10^6	1.4×10^6	100

Table 3.2. Stability of pDiGc in *S.* Typhimurium *in vivo*. The proportion of *S.* Typhimurium that retained pDiGc in the spleens of mice after 48 h post-i.p. inoculation. Plasmid retention was determined by green fluorescence expression after growth on LB agar.

3.2.4.2. Development of alternative reporter systems for murine studies

In light of the limitations of *S.* Typhimurium carrying pDiGc for *in vivo* work, I developed modified reporter systems to overcome these issues. To avoid any detrimental effects of the pBAD18-derived vector backbone, the bacterial expression vector pFPV25 (Cormack *et al.*, 1996), with a ColE1 ori, was chosen as an alternative. This plasmid has a copy number of approximately 15-30 copies per cell. *S.* Typhimurium carrying pFPV25.1 (Valdivia and Falkow, 1996), which carries the *rpsM* promoter upstream of *gfpmut3a* on the pFPV25 vector backbone, is widely used in *S. enterica* research and has been shown to grow at a similar rate as the wild-type strain in RAW264.7 macrophages (Knodler *et al.*, 2005). *S.* Typhimurium SL1344 carrying pFPV25.1 has only a mild virulence attenuation (CI of 0.45) after i.p. inoculation (Knodler *et al.*, 2005) and another study, using strain 12023, stated that in terms of lethality and overall bacterial numbers, bacteria carrying pFPV25.1 were indistinguishable from wild-type bacteria in mice 3 days post-i.p. inoculation (Salcedo *et al.*, 2001).

DsRed has been suggested to attenuate the virulence of *S.* Typhimurium in mice (Prof. D. Bumann, personal communication). This could potentially be due to aggregation of the protein within bacterial cells (Jakobs *et al.*, 2000), given that DsRed exists as a tetramer. In addition, although engineered to have faster replication kinetics (Sorensen *et al.*, 2003), experiments carried out in this study showed that the maturation of this protein still requires

several hours (data not shown). Therefore, an alternative red fluorescent protein, mCherry, was chosen. mCherry was derived from wild-type DsRed via targeted mutagenesis (Shaner *et al.*, 2004). However unlike its precursor, mCherry undergoes fast maturation (approximately 15 min) and is monomeric, which should reduce the likelihood of protein aggregation within bacterial cells. The spectral properties of mCherry also make it a good candidate for use in conjunction with GFP (Table 3.1).

Next, to test which fluorescent protein was most suitable as the reporter for replication, different combinations of protein regulation were designed. These consisted of a system to allow either mCherry or GFP dilution and were termed pFCiGc (FPV25 backbone mCherry-inducible GFP-constitutive; Fig. 3.7A) and pFCcGi (FPV25 backbone mCherry-constitutive GFP-inducible; Fig. 3.7B), respectively. In both plasmids, P_{BAD} was the arabinose-inducible promoter and *rpsM* was the constitutive promoter. Another plasmid termed pFCiGi (pFPV25 backbone mCherry-inducible GFP-inducible; Fig. 3.7C) was also designed. This was because our group found that the pDiGi reporter (Fig. 3.4A) could be used as a tool to assess the viability of intracellular *S.* Typhimurium, based on metabolic activity (Helaine *et al.*, 2010). Using pDiGi, bacteria that persisted within macrophages without replicating (Fig. 3.6C) were shown to retain viability by their ability to respond to the presence of arabinose, detected by *de novo* synthesis of DsRed (Helaine *et al.*, 2010). For this reason, I designed an optimised system to assess *S*. Typhimurium viability *in vivo*. Similar to pDiGi, pFCiGi carries the genes encoding mCherry and GFP upstream of an arabinose- and IPTG-inducible promoter, respectively (Fig. 3.7C).

Of the plasmids described above, pFCiGi was constructed first. Experiments with pFCiGi showed that in *S.* Typhimurium, the fluorescence intensity of mCherry was very low when produced following arabinose induction on the pFPV25 backbone (see below). Therefore, mCherry dilution would provide a limited dynamic range of measurement of bacterial replication, since one of the factors that restricts this is the starting fluorescence intensity. For this

reason, I did not continue constructing pFCiGc and only pFCcGi was brought forward for validation.

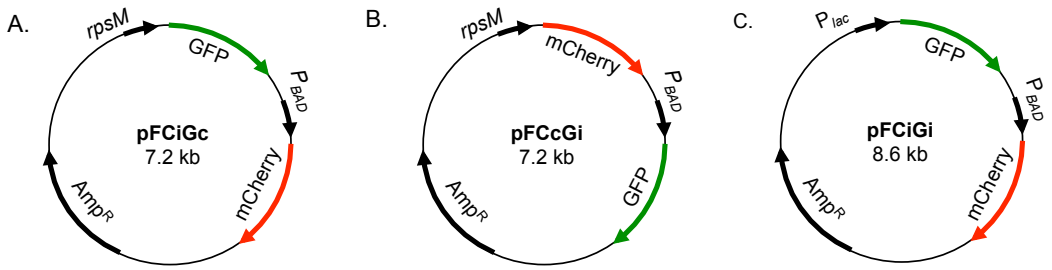


Figure 3.7. Maps of modified reporter plasmids. Map of pFCiGc (A), pFCcGi (B) and pFCiGi (C). mCherry and GFP are under the control of a constitutive (*rpsM)*, arabinose-inducible (P_{BAD}) or IPTG-inducible (P_{lac}) promoter, where indicated. Amp^R denotes the gene for ampicillin resistance. Each vector backbone has been derived from pFPV25.

3.2.5. Suitability of the optimised reporter systems for murine studies

3.2.5.1. Validation of fluorescence dilution using pFCcGi

To check the regulation of fluorescent protein production in *S.* Typhimurium carrying pFCcGi, experiments were conducted as described in Section 3.2.1.3. *S.* Typhimurium carrying pFCcGi that was grown overnight in MM containing arabinose displayed high levels of red and green fluorescence intensity (Fig. 3.8Ai, 3.8Bi). The fluorescence intensity of GFP was similar to that produced constitutively by *S.* Typhimurium carrying pFPV25.1 (Fig. 3.8Aii). Pre-induced *S.* Typhimurium carrying pFCcGi that was subcultured for 8 h in MM without arabinose lost green fluorescence (Fig. 3.8Bii), while maintenance of arabinose prevented this loss (Fig. 3.8Biii). These results were consistent with the expected regulation of GFP production. In addition, pre-induced bacteria carrying pFCcGi that were subcultured in the presence of a bacteriostatic concentration of tetracycline, to block *de novo* protein synthesis and prevent replication, retained high levels of red and green fluorescence intensity (Fig. 3.8Biv), indicating that GFP and mCherry are stable over at least 8 h. Both these fluorescent proteins were shown to be stable for over 72 h in later experiments (see Section 4.2.3.).

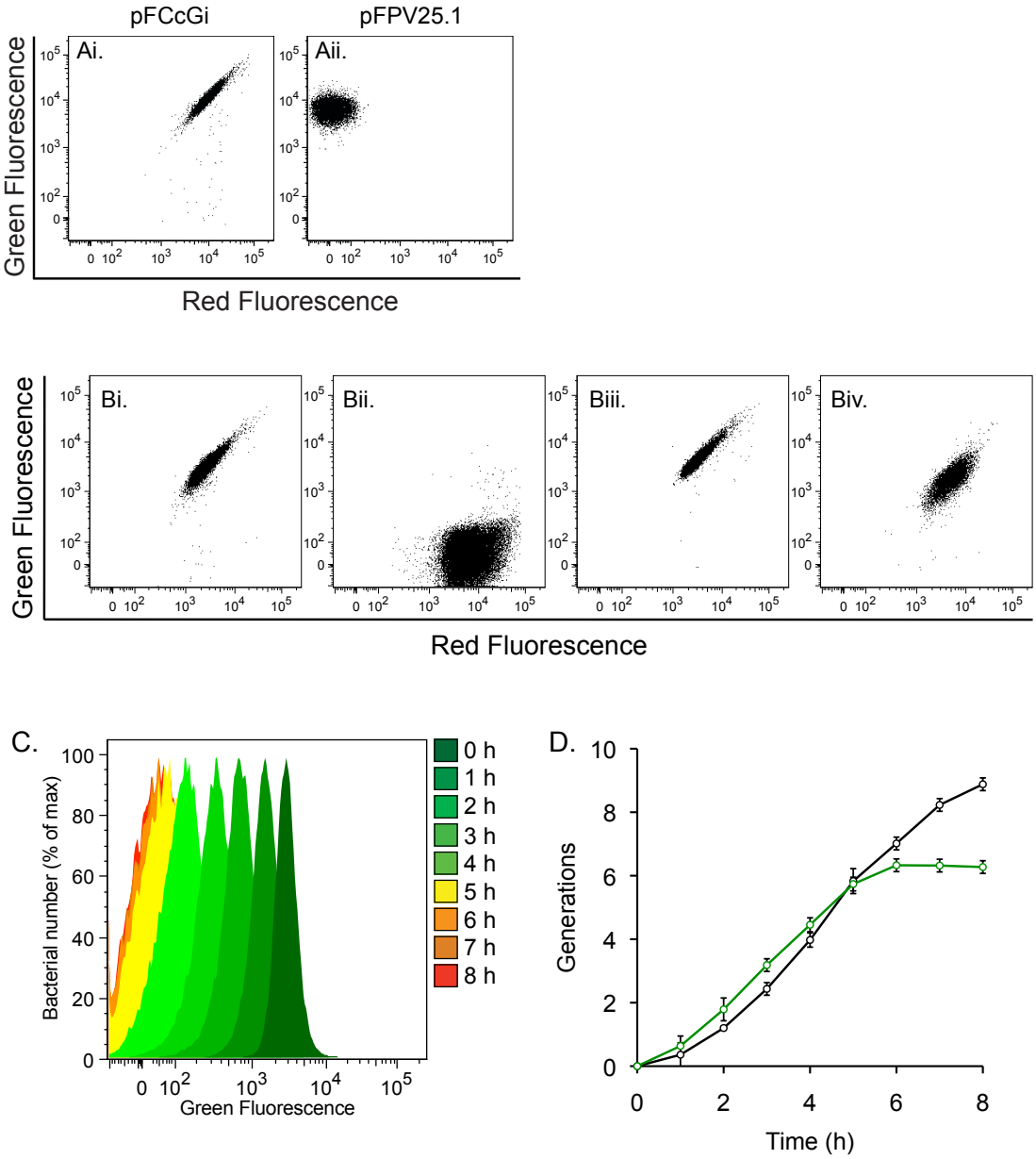


Figure 3.8. Fluorescence dilution of *S* Typhimurium carrying pFCcGi in MM. (A-B) Profiles of red and green fluorescence, detected by flow cytometry, of *S.* Typhimurium carrying pFCcGi (Ai, Bi) or pFPV25.1 (Aii) after growth in MM with arabinose for 16 h and subsequent subculture of *S.* Typhimurium carrying pFCcGi for 8 h in MM without arabinose (Bii), with arabinose (Biii) and with a bacteriostatic concentration of tetracycline (Biv). Data are representative of 2 independent experiments. (C) Profiles of green fluorescence expression in pre-induced *S.* Typhimurium carrying pFCcGi, detected by flow cytometry at hourly intervals, after subculture in MM without arabinose. (D) Bacterial replication measured by CFU (black) and GFP dilution (green). Data represent the mean ± SEM for 2 independent experiments.

Next, experiments were conducted to examine the fluorescence dilution profile of *S.* Typhimurium carrying pFCcGi during bacterial replication in MM and to determine the number of generations that could be measured using this reporter system. Pre-induced *S.* Typhimurium carrying pFCcGi were subcultured in MM without arabinose and bacterial replication was measured by fluorescence dilution and CFU over hourly intervals. A series of normally distributed profiles of green fluorescence intensity was observed (Fig. 3.8C), which were characteristic of bacterial replication measured by DsRed (Fig. 3.3A) and GFP (Fig. 3.4C) dilution in MM. Furthermore, comparison of the bacterial replication profiles obtained by fluorescence dilution and CFU (Fig. 3.8D) demonstrated that fluorescence dilution and CFU were indistinguishable for over at least 6 generations of bacterial growth. Therefore, pFCcGi is a suitable reporter system to facilitate the measurement of *S.* Typhimurium replication by fluorescence dilution.

3.2.5.2. Validation of fluorescence dilution using pFCiGi

Experiments were carried out as described in Section 3.2.1.3. to check the regulation of fluorescence protein production in *S.* Typhimurium carrying pFCiGi. This showed that bacteria grown for 16 h in MM, in the presence of both inducers, produced a similar level of green fluorescence intensity compared to pre-induced bacteria carrying pFCcGi (Fig. 3.9Ai-ii). However, significantly lower levels of red fluorescence intensity were consistently observed (Fig. 3.9Aii). This became even more pronounced after bacterial culture in LB broth (data not shown). One likely explanation for this is the lower fluorescence yield of mCherry compared to GFP (Shaner *et al.*, 2005). When grown for 16 h in MM in the absence of both inducers, *S.* Typhimurium carrying pFCiGi remained non-fluorescent (Fig. 3.9Aiii), confirming the expected regulation of mCherry and GFP.

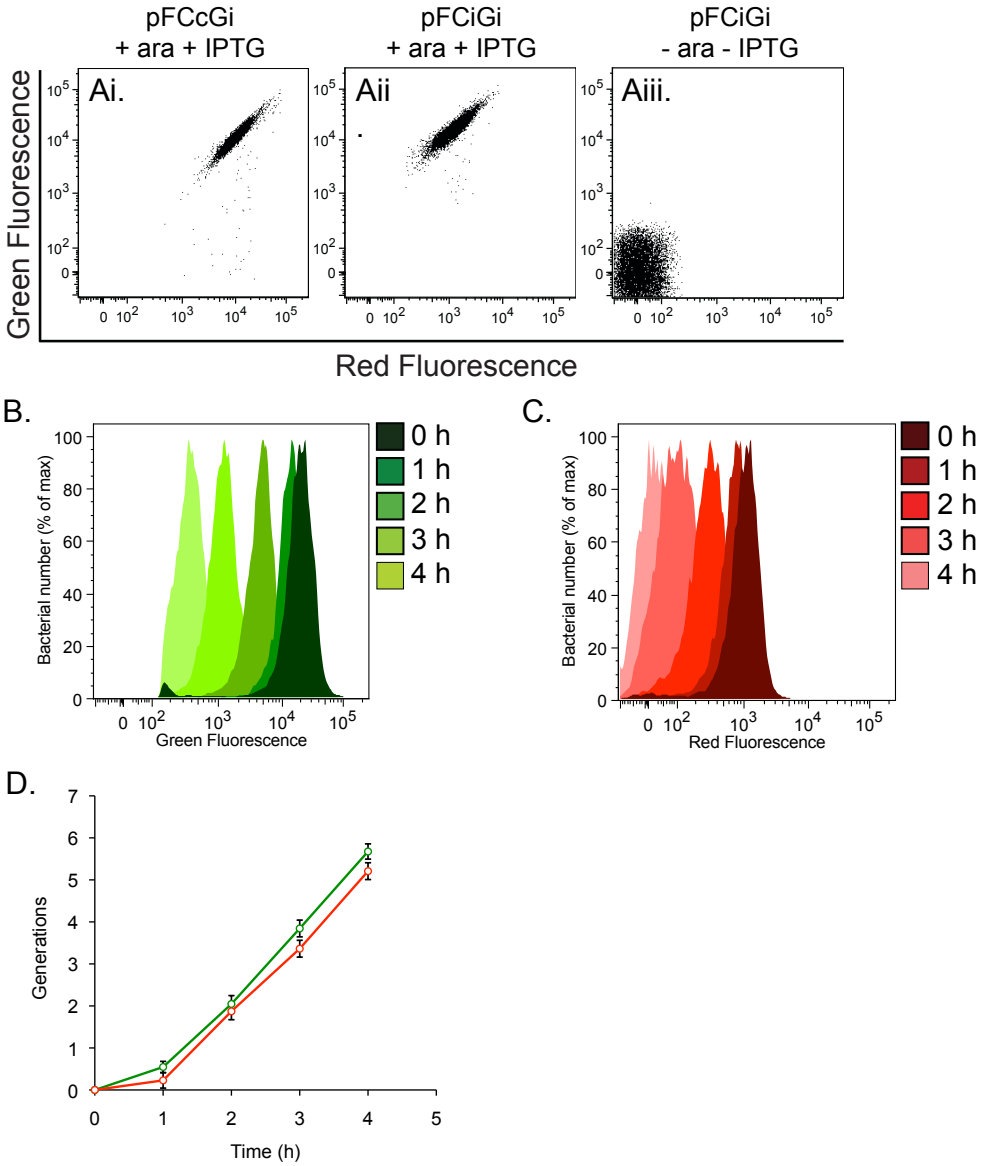


Figure 3.9. Fluorescence dilution of *S* Typhimurium carrying pFCiGi in MM. Flow cytometric detection of green and red fluorescence of *S.* Typhimurium carrying pFCcGi after growth in MM with arabinose (Ai) or carrying pFCiGi after growth in MM with (Aii) or without (Aiii) arabinose and IPTG for 16 h. (B-C) Flow cytometric detection of green (B) and red (C) fluorescence of pre-induced *S.* Typhimurium carrying pFCiGi, after subculture without arabinose and IPTG. Data are representative of 2 independent experiments. (D) Bacterial replication measured by simultaneous dilution of GFP (green) and mCherry (red). Data represent the mean ± SEM for 2 independent experiments.

Next, to confirm the robustness of the fluorescence dilution technique, I also used pFCiGi to establish whether the simultaneous dilution of two fluorescent proteins was comparable. Pre-induced dual fluorescent *S.* Typhimurium carrying pFCiGi were subcultured in MM without arabinose and IPTG and bacterial fluorescence was monitored by flow cytometry over hourly intervals. The fluorescence intensity of both proteins diluted in a uniform manner (Fig.

3.9B-C) and at an approximately equal rate for over 5 generations of bacterial division (Fig. 3.9D). After this time, the level of red fluorescence intensity had reached the limit of detection, preventing further measurements (data not shown). This shows that GFP and mCherry are diluted at equivalent rates within the same cell during bacterial replication. However, these results also demonstrate that the dynamic range of mCherry dilution, in strains carrying pFCiGi, is more limited than GFP.

3.2.5.3. Behaviour of *S.* Typhimurium carrying pFCcGi in MM and different host cells

Having confirmed that the fluorescence expression of *S.* Typhimurium carrying pFCcGi is suitable for use as a reporter system to measure bacterial replication, the fitness cost of this plasmid on *S.* Typhimurium was next investigated. These experiments were conducted with pFCcGi and not pFCiGi, as the aim was to use pFCcGi as a tool to measure *S.* Typhimurium replication. Nevertheless, pFCcGi and pFCiGi share the same vector backbone and fluorescent proteins, therefore it is likely that these two plasmids will possess similar properties.

The influence of pFCcGi on *S.* Typhimurium replication was first examined in MM. Wild-type *S.* Typhimurium and the same strain carrying pFCcGi were grown overnight in MM alone, then subcultured in fresh medium. The increase in CFU was measured over 8 h. This showed that *S.* Typhimurium carrying pFCcGi grew at similar rate compared to the wild-type strain (Fig. 3.10A). Therefore, the vector backbone and constitutive synthesis of mCherry has no significant impact on the replication of *S.* Typhimurium in MM.

Following this, the influence of pFCcGi on *S.* Typhimurium growth in murine macrophage-like cells was assessed. RAW264.7 macrophages were infected with wild-type *S.* Typhimurium or the same strain carrying pFCcGi that had been grown overnight in MM with arabinose. Pre-induced dual fluorescent bacteria were used in these experiments, as these are the conditions under which infections would be carried out when the replication of *S.* Typhimurium

was to be assessed. The infection was allowed to proceed in the absence of arabinose and the fold increase in bacterial CFU between 2 h and 16 h was measured. The fold increase of *S.* Typhimurium carrying pFCcGi was similar to the wild-type strain (Fig. 3.10B), demonstrating that pFCcGi is also well tolerated by *S.* Typhimurium during growth inside macrophages.

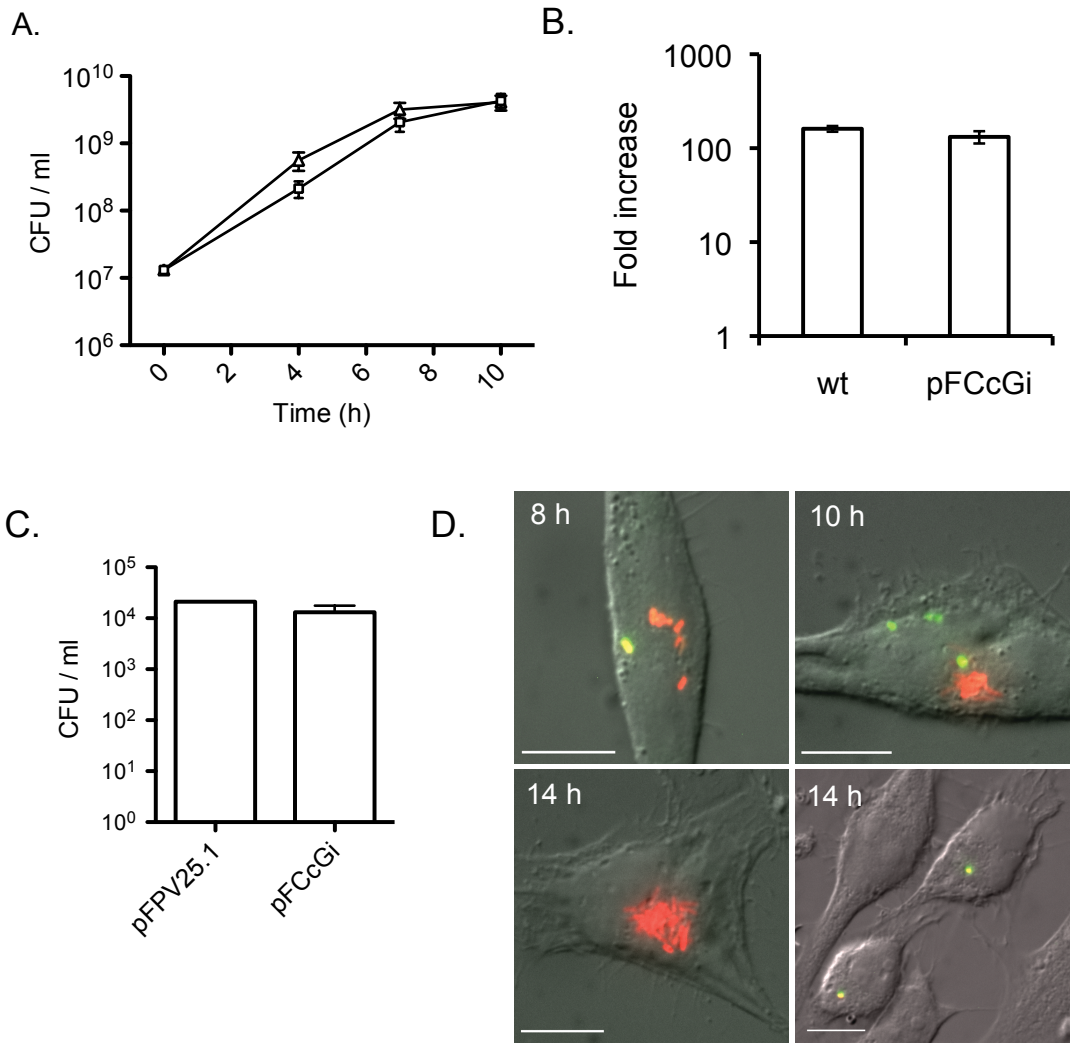


Figure 3.10. Behaviour of *S.* Typhimurium carrying pFCcGi in MM and inside different host cells. (A) Growth of *S.* Typhimurium wild-type (triangles) and the same strain carrying pFCcGi (squares) in MM, determined by CFU enumeration at designated time intervals. (B) Net growth of pre-induced *S.* Typhimurium carrying the pFCcGi compared to the wild-type (wt) strain, in RAW macrophages from 2 h to 16 h post-uptake, determined by fold change in CFU at both time points. Data represent the mean ± SEM 3 independent experiments. (C) Number of intracellular *S.* Typhimurium carrying the indicated plasmids at 2 h post-invasion of HeLa cells. Data represent the mean ± SEM for 2 independent experiments carried out in triplicate. (D) Fluorescence microscopy of pre-induced *S.* Typhimurium carrying pFCcGi at indicated times post-invasion of HeLa cells, showing replicating bacteria (red) and non-replicating bacteria (green / orange). Scale bars: 10 µm.

Next, the ability of *S.* Typhimurium carrying pFCcGi to invade non-phagocytic cells was investigated. Since pFPV25.1 moderately reduces the invasiveness of *S.* Typhimurium (Knodler *et al.*, 2005), the invasion capacity of bacteria carrying pFCcGi was compared to strains carrying pFPV25.1 instead of wild-type *S.* Typhimurium alone. Therefore, wild-type *S.* Typhimurium carrying pFPV25.1 or pFCcGi were grown overnight in MM containing arabinose, then subcultured in LB broth containing arabinose until late logarithmic growth, at which time the SPI-1 T3SS is strongly expressed (Steele-Mortimer *et al.*, 1999). HeLa epithelial-like cells were subsequently infected with these bacteria and the infection was allowed to proceed in the absence of arabinose. Enumeration of intracellular bacteria at 2 h post-invasion showed that *S.* Typhimurium carrying pFCcGi was able to invade these cells at levels similar to bacteria carrying pFPV25.1 (Fig. 3.10C). This shows that pFCcGi carriage does not significantly impair the ability of *S.* Typhimurium to invade non-phagocytic host cells. Interestingly, microscopic analysis of infected HeLa cells also revealed that, as for macrophages, *S.* Typhimurium displayed heterogeneous behaviour, with some bacteria having replicated and others that did not replicate (Fig. 3.10D).

3.2.5.4.cBehaviourr of *S.* Typhimurium carrying pFCcGi in mice

Assessment of the behaviour of *S.* Typhimurium carrying pFCcGi was next extended to the mouse model of typhoid fever. First, plasmid maintenance was checked and this showed that pFCcGi was stably maintained in *S.* Typhimurium in mice over 5 days (Table 3.3). Following this, a CI test of *S.* Typhimurium carrying pFCcGi against the wild-type strain was conducted. Bacteria were grown overnight in MM containing arabinose and then subcultured in LB broth containing arabinose for 2.5 h. Mice were injected i.p. with 5 x 10^5 CFU consisting of wild-type bacteria and those carrying pFCcGi at a 1:1 ratio. After 2 days, mice were sacrificed and bacteria were recovered from the spleens. *S.* Typhimurium carrying pFCcGi had a CI value of 0.44 ± 0.04 (Fig. 3.11A). This showed that pFCcGi conferred a much reduced virulence attenuation on *S.* Typhimurium than pDiGc. The influence of pFPV25.1 on *S.* Typhimurium virulence was also tested under these

conditions. I found that strains carrying pFPV25.1 had a mild virulence attenuation, with a CI of 0.42 ± 0.1 (Fig. 3.11A), which was not significantly different from strains carrying pFCcGi. This is in agreement with another report (Knodler *et al.,* 2005). In addition, single strain infections of mice were carried out using wild-type *S.* Typhimurium or the same strain carrying pFCcGi and the splenic bacterial loads of each strain were compared. Mice were inoculated orally, as this was the desired route of inoculation in later experiments, for the measurement of *S.* Typhimurium replication *in vivo*. Although the number of bacteria carrying pFCcGi in the spleen was less than the wild-type strain on day 4 (Fig. 3.11B), both strains reached a similar level of colonisation on day 6 (Fig. 3.11C). Indeed, *S.* Typhimurium carrying pFPV25.1 has been reported to be as lethal to mice as the wild-type strain (Salcedo *et al.,* 2001). This demonstrates that *S.* Typhimurium carrying pFCcGi is only mildly attenuated in virulence with respect to the wild-type strain and furthermore, these findings indicate that *S.* Typhimurium carrying pFCcGi were able to successfully penetrate the intestinal wall and replicate to reach high loads in the spleens of mice.

Experiment	Total number of bacteria (CFU / spleen)	Number of bacteria carrying pFCcGi (CFU / spleen)	pFCcGi stability (%)
1	6.0×10^5	6.0×10^5	100
2	7.0×10^5	7.0×10^5	100

Table 3.3. Stability of pFCcGi in *S.* Typhimurium in mice. The proportion of wild-type *S.* Typhimurium that retained pFCcGi in the spleens of mice after 5 days p.i. Plasmid retention was determined by red fluorescence expression after growth on LB agar. Groups of 2 mice were used in each experiment.

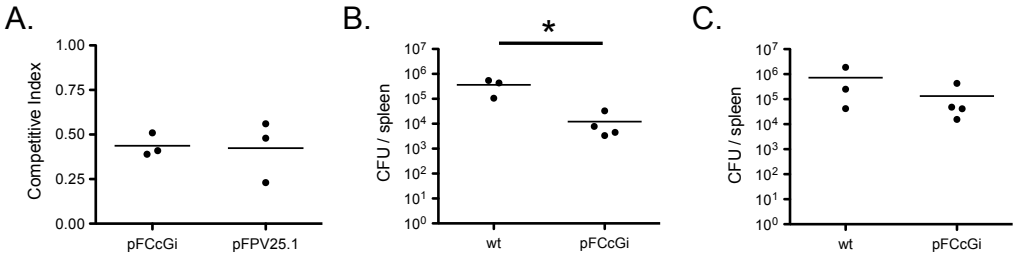


Figure 3.11. Behaviour of *S.* Typhimurium carrying pFCcGi in mice. (A) CI test of *S.* Typhimurium carrying pFCcGi against the wild-type strain after 2 days post-i.p. inoculation of mice. Each symbol represents the mean from independent experiments, in which 5 mice were used per group. (B-C) Bacterial load in the spleen after 4 days (B) or 6 days (C) post-i.g. inoculation of mice with single strains of *S.* Typhimurium wild-type (wt) or the same strain carrying pFCcGi. Each symbol represents the mean from independent experiments, in which 3 mice were used per group. Data were analysed using a paired one-tailed student's *t* test and an asterisk (*) indicates data that is significantly different (p <0.05) compared to the wild-type.

3.3. Summary

In this chapter, I described the use of pDiGc to demonstrate that fluorescence dilution is a suitable tool to quantify bacterial replication directly at the mean population level. This technique also revealed that intracellular populations of *S.* Typhimurium were heterogeneous with respect to replication. In RAW264.7 macrophages, bacterial replication is rapid and homogeneous, with the exception of a sub-population of bacteria that do not replicate. In this chapter, I also described the development of an alternative plasmid, pFCcGi, that is a suitable reporter system for the measurement of *S.* Typhimurium replication by fluorescence dilution. Importantly, this plasmid can overcome the invasion deficiency and virulence attenuation of *S.* Typhimurium caused by carriage of pDiGc. *S.* Typhimurium carrying pFCcGi is virulent in susceptible mice after i.g. inoculation and colonises their spleens almost as efficiently as the wild-type strain. An additional system, pFCiGi was also developed as a tool to assess bacterial viability *in vivo*. Both these modified systems, pFCcGi and pFCiGi, can now be used to study the replication dynamics and viability, respectively, of *S.* Typhimurium during orally-induced systemic disease in mice.

4. VALIDATION OF FLUORESCENCE DILUTION AS A TOOL TO MEASURE BACTERIAL REPLICATION *IN VIVO*

4.1. Introduction

In Chapter 3, I described the development of an optimised plasmid-based reporter system, pFCcGi, to allow the characterisation of the replication dynamics of *S.* Typhimurium during orally-induced murine salmonellosis. Prior to embarking on such investigations, experiments were carried out to validate the use of fluorescence dilution as a tool to measure bacterial replication in mice. This was because animal models are considerably more complex than tissue culture models. More variables exist that could compromise the use of bacterial fluorescence as a reporter of replication, namely the potential basal induction of inducible fluorescent proteins *in vivo*, exposure to acid in the stomach, and oxygen deprivation in the intestinal lumen.

4.2. Results

4.2.1. Identification of *S.* Typhimurium in infected tissue homogenates by red fluorescence emission

Prior to conducting experiments in mice with *S.* Typhimurium, an appropriate means to identify bacteria from infected host tissue debris was established. This was carried out with *S.* Typhimurium carrying pFCcGi that constitutively produce red fluorescence. It has been shown that tissue debris can emit a significant amount of autofluorescence, both in the green and red spectra (Bumann, 2002). Unless bacterial fluorescence intensity is relatively high, bacteria cannot be adequately distinguished from background autofluorescent molecules. Red fluorescent bacteria cultured in MM or LB broth can be easily identified by flow cytometry, according to size (forward scatter; FSC) and granularity (side scatter; SSC) and high red fluorescence (Fig. 4.1Ai-ii). In uninfected mice, no high red fluorescent particles of a similar size and granularity to bacteria were detected (Fig. 4.1Bi-ii). However, in the tissues of mice infected with *S.* Typhimurium carrying pFCcGi, a distinct high red

fluorescent population was observed after gating on particles with similar FSC and SSC properties to bacteria (Fig. 4.1Ci-ii). Therefore, constitutive red fluorescence of *S.* Typhimurium carrying pFCcGi is an effective means of identifying bacteria from autofluorescent host tissue debris.

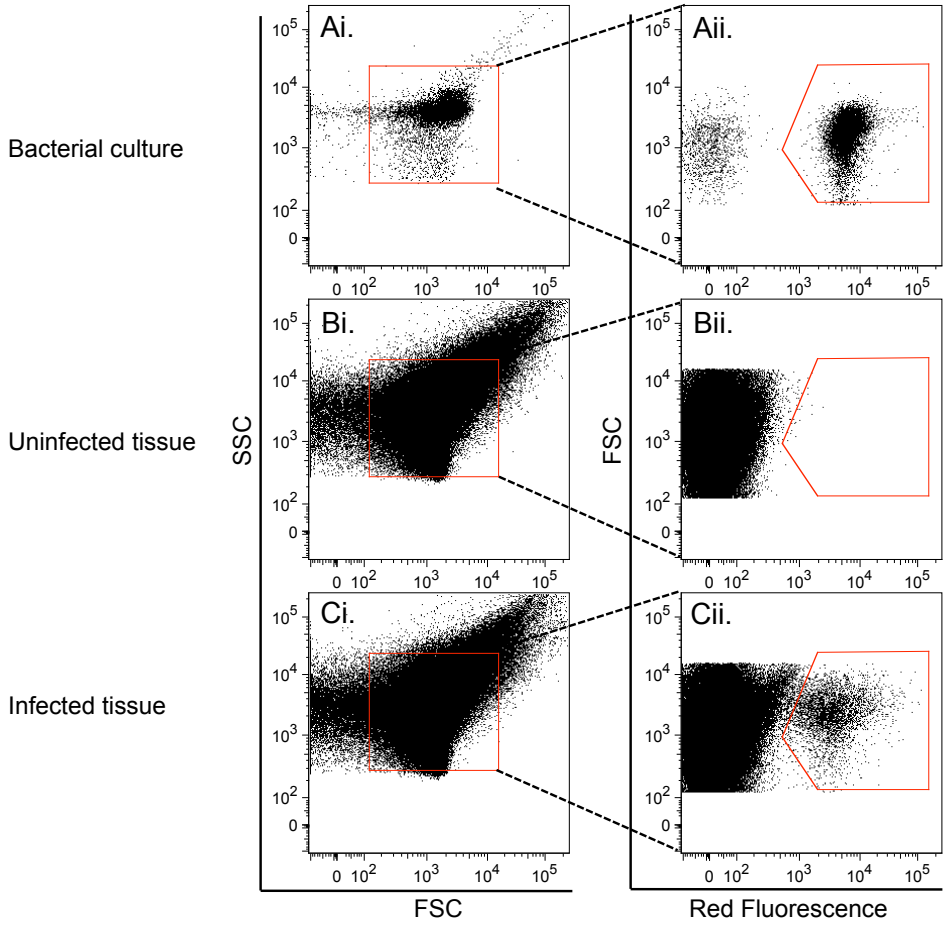


Figure 4.1. Identification of *S.* Typhimurium in murine tissues by red fluorescence emission. Flow cytometric detection of pre-induced *S.* Typhimurium carrying pFCcGi in liquid culture (A), uninfected murine tissue (B) or infected murine tissue (C). Bacteria are identified by first gating on fragments with similar FSC and SSC properties to *S.* Typhimurium (Ai, Bi, Ci), followed by a second gating on particles that emit either high levels of red fluorescence intensity (Aii, Bii, Cii).

4.2.2. Investigating the levels of arabinose in mice

To use GFP fluorescence as a reporter of replication, it is important to ensure that the production of this protein by *S.* Typhimurium carrying pFCcGi is under strict control. In earlier experiments conducted *in vitro*, this was confirmed by culturing bacteria in the absence and presence of arabinose. However, given that arabinose is a natural sugar, it is possible that it could be present in

digested food in the gastrointestinal tract of mice or as metabolised products in the blood. Therefore, experiments were conducted to establish whether there were sufficient levels of arabinose available in murine tissues to induce fluorescent protein production. Mice were inoculated orally with *S.* Typhimurium carrying pFCcGi that had not been incubated with arabinose prior to inoculation. Bacteria were then recovered from the PP and MLN at 48 h p.i. and flow cytometry was used to examine their levels of green fluorescence compared to bacteria in the inoculum. There was no significant increase of green fluorescence intensity in bacteria that had been recovered from infected mice compared to those in the inoculum (Fig. 4.2A). Therefore, in murine tissues, arabinose is not present at levels sufficient to induce P_{BAD}-mediated fluorescence production in *S.* Typhimurium carrying pFCcGi.

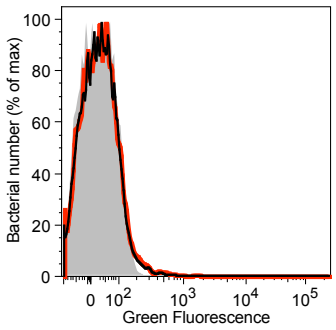


Figure 4.2. Arabinose induction of intrabacterial GFP in mice. Mice were inoculated orally with uninduced *S.* Typhimurium carrying pFCcGi and sacrificed after 48 h. The red and green fluorescence emission of bacteria in the inoculum (grey) and after recovery from the PP (black) and MLN (red) was detected by flow cytometry.

4.2.3. Fluorescence stability of intrabacterial mCherry and GFP under acidic and anaerobic conditions *in vitro*

During passage through the gastrointestinal tract, *S.* Typhimurium is exposed to many stressful conditions, some of which could alter the levels of intrabacterial GFP and mCherry fluorescence intensity. This is a particularly important consideration if fluorescence dilution is to be used as a tool to measure bacterial replication *in vivo*, since information about replication is based exclusively on the decrease of bacterial fluorescence intensity. Therefore, a change in fluorescence due to factors other than replication could lead to misinterpretation of the results.

Bacteria entering the stomach are exposed to gastric acid, formed mainly of hydrochloric acid. This can create environments as acidic as pH 1 or 2 and

acts as an effective barrier to infection. Short chain fatty acids present in the intestine can also exert acid stress on *S.* Typhimurium in the intestine (Bohnhoff *et al.*, 1964). With this in mind, it is known that fluorescent proteins can be sensitive to pH. Indeed, the fluorescence intensity of purified GFP (Doherty *et al.*, 2010), and cytosolic GFP in *Streptococcus gordonii* (Hansen *et al.*, 2001), decreased when placed in a gradually lower ambient pH from ≥ 7.0 to ≤ 4.5. Indeed, the fluorescence intensity of GFP can act as a pH indicator (Llopis *et al.*, 1998). In contrast, mCherry is reported to be more resistant to fluctuations in pH, as the purified protein maintained a relatively constant fluorescence intensity when resuspended in a range of acidic to alkaline buffers (Doherty *et al.*, 2010). Therefore, experiments were conducted to examine whether culturing *S.* Typhimurium in a very acidic environment, to mimic the conditions that bacteria might be exposed to in the stomach, would alter the fluorescence intensity of intrabacterial GFP and mCherry.

S. Typhimurium carrying pFCcGi emits high levels of green and red fluorescence when grown overnight in MM pH 5.0 in the presence of arabinose (Fig. 3.8Ai). When these dual fluorescent bacteria are then exposed to an ambient pH of 2.0, in the presence of a bacteriostatic concentration of tetracycline to inhibit protein synthesis and prevent replication, all bacteria rapidly (within 5 min) lost green fluorescence (Fig. 4.3A). This pH also caused 100% bacterial death, as determined by a subsequent lack of growth on LB agar plates (data not shown). Therefore, it was unsurprising that the green fluorescence loss could not be rescued when bacteria were returned to a medium of neutral pH (Fig. 4.3B). However, it was interesting that a decrease in red fluorescence was never observed (Fig. 4.3A), indicating that mCherry remains highly stable after exposure of bacteria to ambient acidic conditions and can maintain fluorescence even in dead bacteria.

Another factor that could potentially alter the fluorescence of intrabacterial GFP and mCherry *in vivo* is the availability of oxygen. This was of particular concern in the gastrointestinal tract, where oxygen levels have been shown to

progressively decrease from the stomach to the rectum in living mice (He *et al.*, 1999). In addition, it is generally believed that the lumen of the intestine represents an anaerobic environment, with oxygen rich regions localised to small zones above the microvilli (Marteyn *et al.*, 2010). Both GFP and mCherry require molecular oxygen for maturation of the fluorophore (Heim *et al.*, 1994, Remington, 2006, Marteyn *et al.*, 2010, Doherty *et al.*, 2010). However, GFP and mCherry maturation can occur in very low levels of oxygen (Hansen *et al.*, 2001, Carroll *et al.*, 2010, Marteyn *et al.*, 2010) and partial fluorophore formation was observed in the caeca of mice, when GFP was synthesised by *S.* Typhimurium *in vivo* (Rollenhagen and Bumann, 2006). It is unclear whether a continued supply of oxygen is required to maintain the stability of fluorescence after complete maturation of the protein in the presence of oxygen. This is an important point to consider, as fluorescence dilution relies entirely on the stability of a *pre-formed* fluorescent protein. Therefore, experiments were conducted to establish whether oxygen withdrawal might affect the stability of intrabacterial GFP and mCherry after prior synthesis and maturation of the proteins in oxygen replete conditions. *S.* Typhimurium carrying pFCcGi was grown overnight in medium with arabinose under aerobic conditions at 37°C. Arabinose was then washed out and a bacteriostatic concentration of tetracycline was added, to block further protein synthesis and prevent replication. From this point onwards, half the culture was kept in aerobic conditions and the other half was placed in anaerobic conditions at 37°C. The fluorescence intensity of GFP and mCherry was monitored by flow cytometry over 3 days. In aerobic conditions, the fluorescence of both GFP and mCherry was approximately 100% stable over 72 h (Fig. 4.3C-D), indicating that in *S.* Typhimurium these proteins have very long half-lives. However, when placed under anaerobic conditions *in vitro*, GFP lost green fluorescence after 24 h (Fig. 4.3C), while mCherry fluorescence remained relatively unaffected (Fig. 4.3D). This shows that after a period of at least 24 h after the removal of oxygen, the fluorophore of mature GFP becomes unstable. Furthermore, these changes were permanent, as no recovery of green fluorescence was observed after subsequent incubations in fully aerated media at 37°C for up to 24 h (Fig. 4.3E).

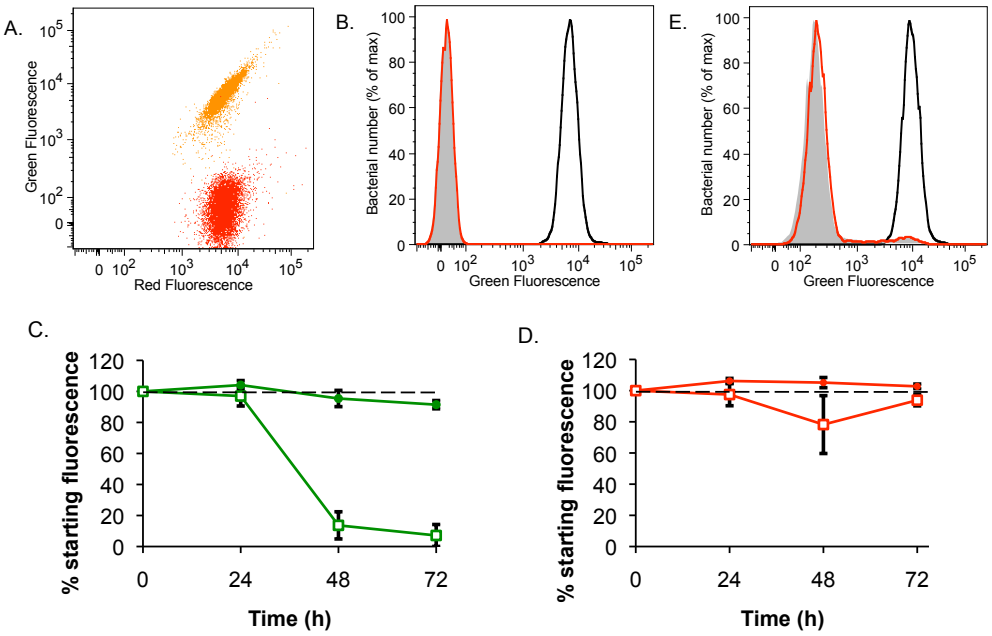


Figure 4.3. Fluorescence stability of intrabacterial mCherry and GFP under acidic and anaerobic conditions *in vitro*. (A-B) Flow cytometric detection of fluorescence expression in pre-induced *S*. Typhimurium carrying pFCcGi after exposure to ambient acidic conditions, in the absence of arabinose and in a bacteriostatic concentration of tetracycline. (A) Red and green fluorescence of bacteria before (orange) and after exposure to pH 2.0 for 5 min (red). (B) Green fluorescence of pre-induced bacteria before (black) and after exposure to pH 2.0 for 2 h (red), followed by culture of bacteria in PBS for a further 24 h (grey). Data are representative of 3 independent experiments. (C-E) Flow cytometric detection of fluorescence expression in pre-induced *S*. Typhimurium carrying pFCcGi after exposure to anaerobic conditions, in the absence of arabinose and in a bacteriostatic concentration of tetracycline. (C-D) Percentage loss of green (C) and red (D) fluorescence intensity compared to the intensity of fluorescence at t_{0h}, in pre-induced *S.* Typhimurium carrying pFCcGi, during culture in the presence (filled triangles) of absence (open squares) of oxygen. Data represents the mean ± SEM for 3 independent experiments. (E) Green fluorescence of pre-induced bacteria before (black) and after culture in anaerobic conditions for 48 h (red), followed by culture of bacteria in aerobic conditions for a further 24 h (grey). Data are representative of 3 independent experiments.

Together, these results show that in contrast to mCherry, intrabacterial GFP is more unstable when bacteria are exposed to ambient anaerobic and acidic conditions and this becomes apparent by loss of green fluorescence. These findings also demonstrate that intrabacterial GFP is more sensitive to an ambient drop in pH, rather than the removal of oxygen. In light of this, mCherry seems more suitable than GFP, as the dilutable fluorescence reporter to measure bacterial replication *in vivo*. However, unfortunately the low fluorescence yield of mCherry from pFCiGi (Fig. 3.9Aii) significantly restricts the number of bacterial divisions that can be measured by fluorescence dilution. The fluorescence intensity of mCherry is particularly low, when *S.* Typhimurium is grown in LB broth compared to MM (data not

shown). Therefore, the dynamic range of measurement using mCherry dilution would be even more restricted in murine studies, since bacteria must be subcultured in LB broth prior to inoculation of mice.

4.2.4. Using the levels of GFP fluorescence intensity and intracellular protein to detect replication-independent loss of fluorescence

4.2.4.1. Correlation between levels of GFP fluorescence intensity and intracellular protein

To test whether a replication-independent loss of green fluorescence would occur *in vivo*, I developed the following hypothesis: if loss of fluorescence is exclusively due to replication, then the decrease in fluorescence intensity will correlate with a decrease in the amount of intracellular fluorescent protein (Fig. 4.4). This is because the pre-formed pool of fluorescence protein halves between bacteria as they divide (Fig. 1.6B). Accordingly, if loss of fluorescence occurs due to factors other than replication, then assuming the level of intracellular protein remains unaffected, the levels of fluorescence intensity and intracellular fluorescent protein will not correlate. By permeabilising bacteria and labelling with fluorescently-labelled antibodies specific to GFP, this should provide a means to quantify the levels of GFP fluorescence and intracellular protein using flow cytometry.

Prior to testing this hypothesis in fluorescent bacteria recovered from mice, I conducted experiments to determine whether the two scenarios of replication-dependent and independent fluorescence loss could be distinguished during controlled conditions *in vitro*.

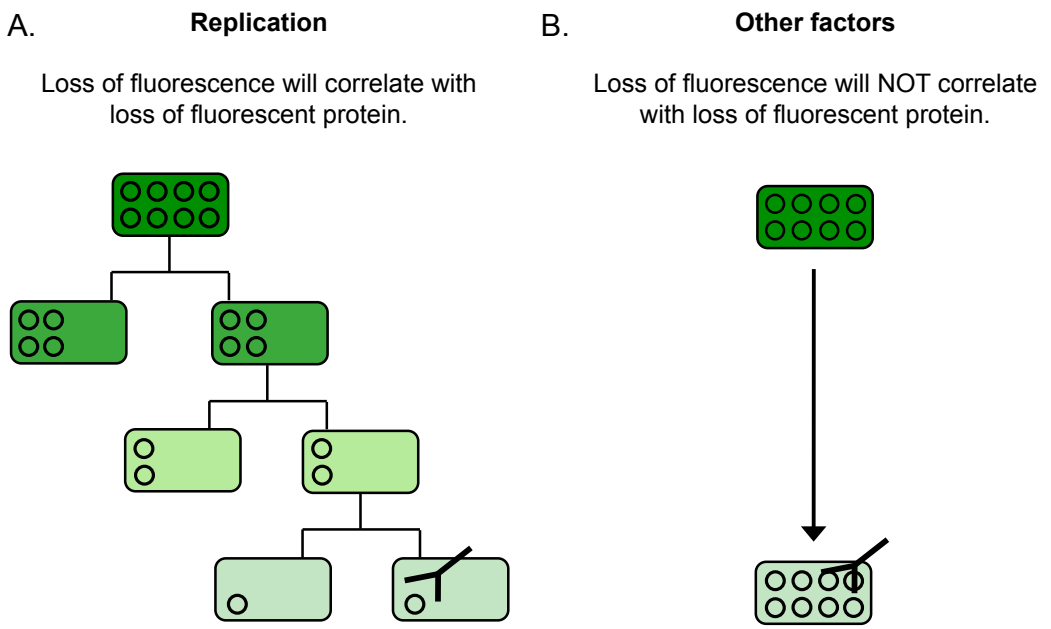


Figure 4.4. Level of green fluorescence intensity and intracellular protein in bacteria under different situations. Diagram showing the hypothesised relationship between intrabacterial GFP and green fluorescence under different scenarios. (A) If green fluorescence loss is due to replication, then the decrease in fluorescence intensity will correlate with a decrease in the level of intracelluar fluorescent protein. (B) However, if loss of green fluorescence is due to factors other than replication, the decrease in green fluorescence intensity will not correlate with a decrease in intracellular fluorescent protein levels. The amount of intracellular GFP can be detected by antibody labelling.

4.2.4.2. Detection of intracellular GFP following bacterial replication

Firstly, I wished to establish whether anti-GFP labelling of permeabilised bacteria was an effective means to quantify intracellular GFP levels. To do this, wild-type bacteria and the same strain carrying pFCcGi were grown overnight in the presence of arabinose. Bacteria carrying pFCcGi produced both red and green fluorescence and the wild-type strain was non-fluorescent. Following this, bacteria were fixed, permeabilised and labelled with primary anti-GFP antibodies and secondary antibodies conjugated to a far-red fluorescent protein, then analysed by flow cytometry. Bacteria emitting high levels of green fluorescence displayed substantially greater levels of antibody labelling compared to those that were labelled with secondary antibody alone or compared to non-fluorescent wild-type bacteria labelled with both primary and secondary antibodies (Fig. 4.5A). This shows that anti-GFP labelling is specific. Furthermore, although levels of intracellular protein labelling were

sometimes variable, in most cases up to 100% of bacteria that expressed green fluorescence, also showed high levels of antibody labelling (Fig. 4.5A). This confirms that anti-GFP antibodies can efficiently detect intracellular protein.

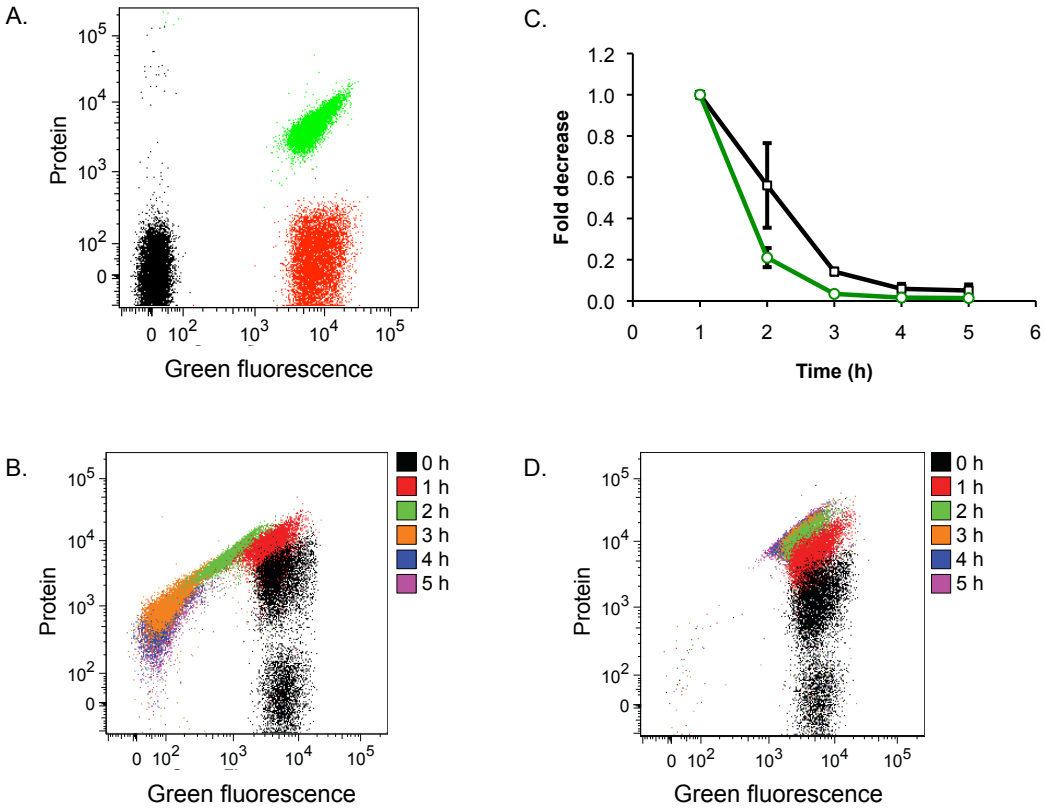


Figure 4.5. Detection of intracellular GFP following bacterial replication. (A) Flow cytometric fluorescence detection of wild-type (black) or pre-induced *S.* Typhimurium carrying pFCcGi (green) after labelling with primary and secondary antibodies and pre-induced *S.* Typhimurium carrying pFCcGi after labelling with secondary antibody only (red). (B and D) Flow cytometric fluorescence detection of pre-induced *S.* Typhimurium carrying pFCcGi at hourly intervals during subculture in LB broth without arabinose and in the absence (B) or presence (D) of a bacteriostatic concentration of tetracycline. Bacteria were labelled with primary and secondary antibodies to detect intracellular GFP. (C) The fold decrease of bacterial green fluorescence intensity (green) and intracelluar protein (black) during subculture in LB broth was calculated at each time-point. Data represent the mean ± SEM for 3 independent experiments.

Next, experiments were carried out to verify that the level of intracellular GFP decreased in parallel with the decrease of green fluorescence intensity during bacterial replication. Dual fluorescent *S.* Typhimurium carrying pFCcGi were subcultured in LB broth alone (to allow replication to proceed) or LB broth supplemented with a bacteriostatic concentration of tetracycline (to prevent replication). At various times, bacteria were fixed, permeabilised, labelled with

anti-GFP antibodies and analysed by flow cytometry. A small proportion of the starting culture often had lower levels of antibody labelling, despite emitting high levels of green fluorescence (Fig. 4.5B and D). The reason for this is unclear, considering that equivalent numbers of bacteria were labelled at each time point and all samples were treated identically. It is possible that alterations in cell structure associated with being in stationary phase may affect the efficiency of bacterial permeabilisation. In any case, bacteria were always efficiently labelled 1 h after subculture. When replication was allowed to proceed, a decrease of intracellular protein levels was observed and this roughly paralleled the decrease of green fluorescence intensity, at approximately the same rate (Fig. 4.5B-C). However, when replication was prevented, a decrease in green fluorescence intensity and intracellular protein levels was not observed (Fig. 4.5D). Therefore, the decrease in intracellular GFP correlates with the decrease in green fluorescence intensity during bacterial replication *in vitro*. In addition, the increased variation obtained when using protein immunolabelling and detection by flow cytometry to measure replication demonstrates that this method is not as sensitive as monitoring fluorescence intensity. This is not surprising, considering that detection of intracellular protein is indirect and requires several intermediate processing steps.

4.2.4.3. Detection of intrabacterial GFP following exposure to anaerobic or acidic conditions

Next, I carried out experiments to test a second hypothesis: that intracellular protein levels would not correlate with fluorescence intensity if fluorescence was lost due to replication-independent factors, such as anoxia or low pH. Pre-induced *S.* Typhimurium carrying pFCcGi were permeabilised and labelled for intracellular GFP before and after exposure to anaerobic conditions for up to 72 h (Fig. 4.6A) or acidic conditions (pH 2.0) for 5 min (Fig. 4.6B). In case these conditions caused conformational changes in GFP that masked or altered the antibody epitopes, I used both mono- and polyclonal anti-GFP antibodies that were capable of recognising denatured GFP. Throughout the experiment, bacteria were kept in medium containing a

bacteriostatic concentration of tetracycline to prevent bacterial replication. Levels of green fluorescence intensity and intracellular GFP were detected by

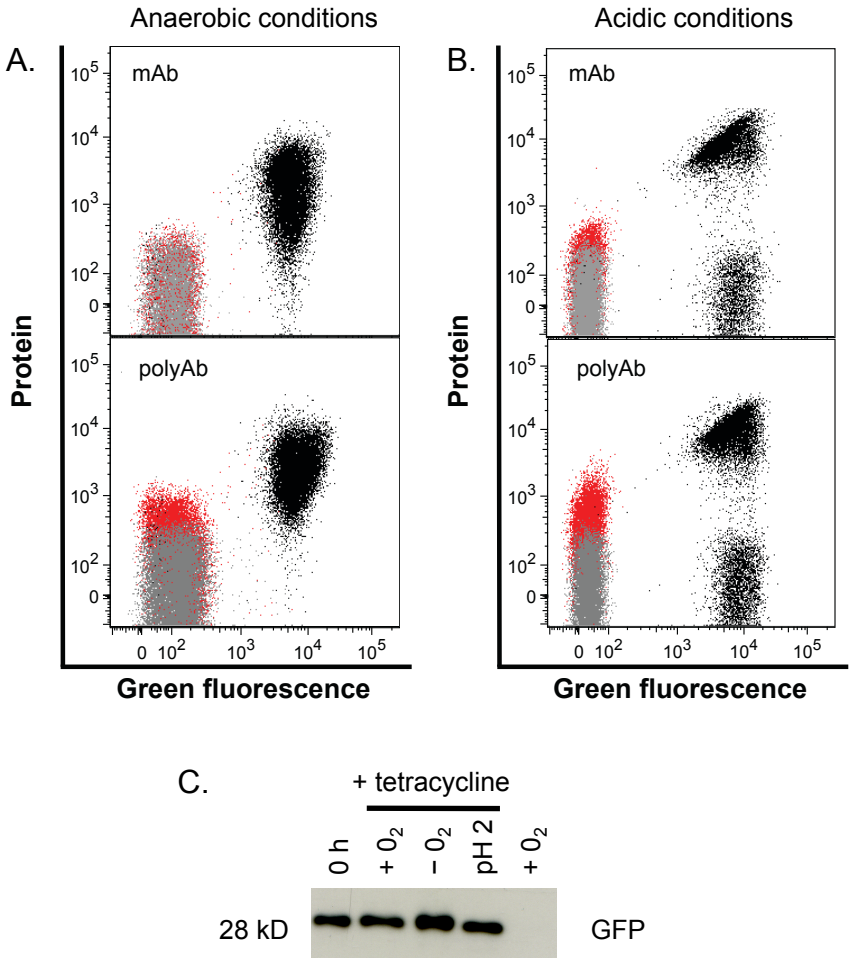


Figure 4.6. Detection of intracellular protein following exposure to anaerobic and acidic conditions. (A-B) Flow cytometric fluorescence detection of pre-induced *S.* Typhimurium carrying pFCcGi before (black) and after (red) exposure to anaerobic conditions for 72 h (A) or pH 2.0 for 5 min (B). Bacteria were labelled with monoclonal (top panel) or polyclonal (bottom panel) anti-GFP antibodies. Bacteria labelled with secondary antibody only are also shown (grey). (C) Immunoblotting of intrabacterial GFP in pre-induced *S.* Typhimurium carrying pFCcGi, after subculture in aerobic or anaerobic conditions for 72 h or exposure to pH 2.0 for 5 min, in a bacteriostatic concentration of tetracycline. Pre-induced bacteria that were also grown in aerobic conditions for 16 h without tetracycline (last lane). Data are representative of at least 3 independent experiments.

flow cytometry.

As expected, the levels of both green fluorescence intensity and intracellular protein were high after culture in non-stressful conditions (black, Fig. 4.6A-B). However, when green fluorescence was lost after exposure of bacteria to both acidic and anaerobic conditions, intrabacterial GFP could no longer be

detected by permeabilisation and antibody labelling with either mono- or polyclonal antibodies (red, Fig. 4.6A-B). This was evident because the level of antibody labelling in these bacteria was at a similar level to those labelled with secondary antibody alone (grey, Fig. 4.6A-B). This suggested that the loss of green fluorescence observed under ambient anaerobic or acidic stress could be due to degradation of intracellular GFP. However, it was possible that after exposure to acidic and anaerobic conditions, intracellular GFP was still present but no longer detectable by anti-GFP antibody labelling. This was suggested by the inefficient antibody labelling of highly green fluorescent bacteria taken from stationary-phase cultures (Fig. 4.5B and D and Fig. 4.6B). Therefore, this assay was not able to distinguish between replication-dependent or independent loss of bacterial fluorescence. In both scenarios, bacteria that had lost green fluorescence also appeared to have decreased levels of intracellular protein.

In an attempt to provide a better means to distinguish between replication-dependent and independent loss of green fluorescence, an independent method to detect intracellular GFP was used. Instead of permeabilising bacteria and detecting GFP *in situ*, bacteria were lysed to release their intracellular proteins, so as to improve access of antibodies to GFP. Samples containing pre-induced bacteria, carrying pFCcGi, cultured in MM under different conditions were normalised to an OD_{600} of 0.01 to ensure that the same concentration of bacteria were analysed in each condition. Interestingly, this showed that GFP is indeed still present in bacteria after loss of fluorescence in anaerobic or acidic conditions (Fig. 4.6C), indicating that this protein was not degraded, but rather, was not detected by anti-GFP antibody while contained within the bacteria. In addition, pre-induced *S.* Typhimurium carrying pFCcGi was subcultured in aerobic conditions for 16 h without tetracycline, to allow replication to proceed under oxygen-rich conditions. As expected, the absence of intracellular GFP was confirmed within these bacteria that had replicated, demonstrating the specificity of the antibodies for GFP (Fig. 4.6C).

Therefore, these results are consistent with the hypothesis that the levels of intracellular GFP decrease only if green fluorescence is lost during bacterial replication. Importantly, these findings also show that protein immunoblotting of bacterial lysates, rather than flow cytometric detection of intracellular protein in bacterial suspensions, is a suitable means to detect intracellular GFP in replication-independent conditions.

4.2.5. Using pFCiGi to allow direct comparison of mCherry and GFP dilution

Although I established protein immunoblotting of bacterial lysates as a suitable method to identify replication-independent loss of GFP fluorescence, unfortunately this technique was not applicable for analysis of *S.* Typhimurium fluorescence *in vivo*. This was because preliminary experiments revealed that bacteria recovered from murine tissues often displayed a heterogeneous profile of green fluorescence intensity and protein immunoblotting can only provide analysis at the global population level. For this reason, I decided to use an alternative approach, which was capable of detecting population variation of green fluorescence, to determine whether replication-independent bacterial fluorescence loss occurs *in vivo*.

The previous experiments highlighted the increased stability of intrabacterial mCherry compared to GFP after exposure to extremely low ambient pH (Fig. 4.3A) and in the complete absence of oxygen (Fig. 4.3D), two factors that could potentially alter fluorescent protein stability *in vivo*. Therefore, I took advantage of the increased stability of mCherry to address whether GFP was unstable *in vivo*. To do this, pFCiGi was used, as this system allows the stability of intrabacterial mCherry and GFP to be compared directly in the same bacterial cell. Given that mCherry and GFP were shown to dilute at a similar rate in *S.* Typhimurium carrying pFCiGi during replication under non-stressful conditions *in vitro* (Fig. 3.9D), the profile of GFP fluorescence should resemble that of mCherry if there is no replication-independent loss of GFP fluorescence. However, if bacteria are exposed to conditions that could alter protein stability, GFP is more likely to be affected compared to mCherry. In

this situation, the fluorescence of GFP should be different from that of mCherry. In addition, by using pFCiGi and expressing both fluorescent proteins from the same plasmid, inter-experiment variation can be avoided. Otherwise, two parallel infections of different mice would have to be conducted, using two bacterial strains producing either inducible-GFP or mCherry from a different plasmid. Furthermore, using pFCiGi also rules out any effects that might be attributed to differences in virulence, which could result from variations in the reporter constructs, if two bacterial strains carrying different plasmids were used.

4.2.5.1. Identification of *S.* Typhimurium in infected tissue homogenates by CSA-1 labelling

Unlike pFCcGi, there is no constitutive fluorescent marker encoded within pFCiGi to enable the detection of bacteria from autofluorescent host tissue fragments. Therefore, these bacteria were labelled with antibody against surface-expressed CSA-1, followed by secondary labelling with antibodies conjugated to a far-red fluorescent dye. To determine whether antibody labelling is a suitable method of identifying *S.* Typhimurium from host-tissue fragments, the efficiency and specificity of anti-CSA-1 antibody labelling was tested using *S.* Typhimurium carrying pFCcGi, which can be easily identified in infected tissue homogenates, based on red fluorescence detection (Fig. 4.1Cii and Fig. 4.7Ai). To do this, mice were inoculated orally with *S.* Typhimurium carrying pFCcGi and sacrificed after 72 h. Various tissues were removed, including the PP and MLN. These were homogenised and fixed, then incubated with primary anti-CSA-1 antibodies and secondary far-red fluorescent-conjugated antibodies and analysed by flow cytometry. Approximately 100% of bacteria, identified by high red fluorescence, were labelled with anti-CSA-1 antibody (Fig. 4.7Aii). Therefore, anti-CSA-1 can label *S.* Typhimurium very efficiently in infected tissue homogenates. However, when gating on all anti-CSA-1 positive events that are of a similar size and granularity to *S.* Typhimurium (Fig. 4.7Bi), only approximately 65% of these simultaneously emitted high levels of red fluorescence (Fig. 4.7Bii), enabling them to be identified unambigously as bacteria. It is likely that non-

specific anti-CSA-1 labelling is due to antibody binding to bacterial debris that are no longer fluorescent. Indeed, other groups also reported high levels of background labelling when using anti-*Salmonella* specific antibodies in infected tissues (Bumann, 2002, Rydstrom and Wick, 2007). This demonstrates that detecting the fluorescence intensity of a constitutively synthesised fluorescent protein is a more effective method of identifying *S.* Typhimurium from autofluorescent host tissue fragments, compared to labelling with anti-CSA-1.

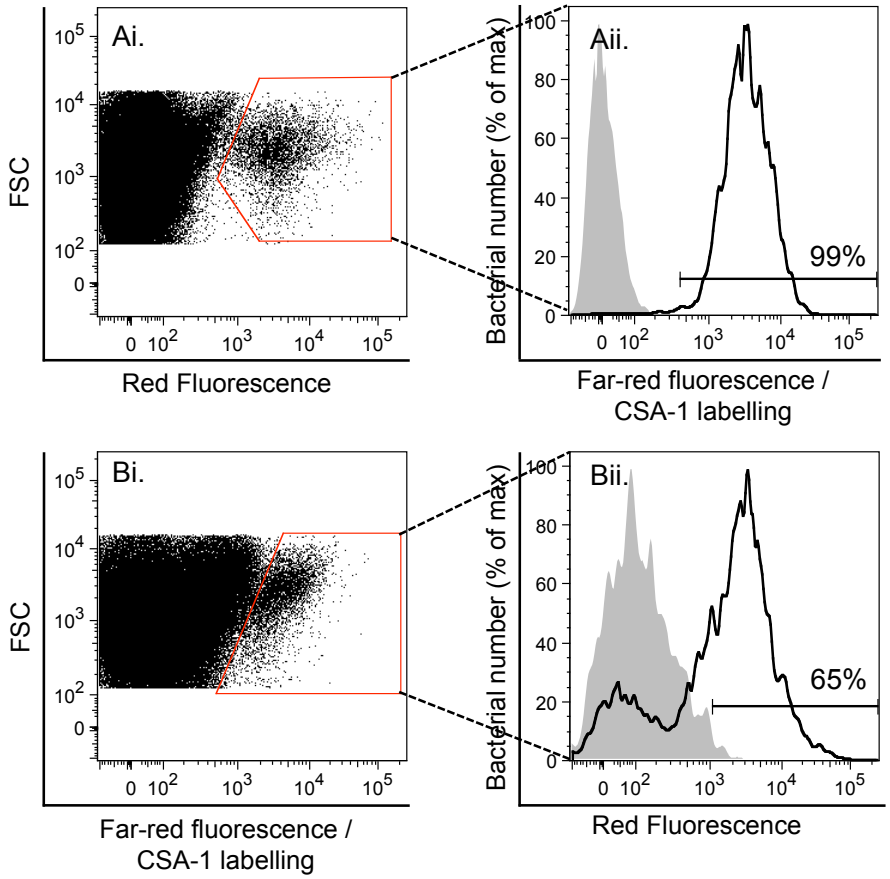


Figure 4.7. Identification of *S.* Typhimurium in murine tissues by CSA-1 labelling. Flow cytometric detection of pre-induced *S.* Typhimurium carrying pFCcGi in infected murine tissue, after labelling with primary anti-CSA-1 antibodies and secondary antibodies conjugated to a far-red fluorescent dye. Bacteria are identified from autofluorescent host tissue fragments of a similar size by high red fluorescence (Ai) or high far-red fluorescence (Bi) emission. Bacteria that have been labelled with anti-CSA-1 antibodies emit high intensities of far-red fluorescence (black, Aii), compared to unlabelled bacteria in the inoculum (grey, Aii). Alternatively, CSA-1 labelled particles, which represent bacteria, emit high intensities red fluorescence (black, Bii), compared to CSA-1 labelled uninfected murine tissue (grey, Bii). Note, data in (Ai) has been reproduced from Fig. 4.1Cii.

The high degree of non-specific labelling with anti-CSA-1 antibody presented a problem for experiments requiring the use of pFCiGi, as this method could not be used to reliably distinguish *S.* Typhimurium from autofluorescent host tissue fragments. However, a means to overcome this was developed. Anti-CSA-1 labelling was used as a first round of gating to eliminate the majority of autofluorescent host tissue fragments. Following this, further non-specific labelling was excluded by gating only on events with very high levels of red fluorescence, which should represent intact bacteria. Therefore, only bacteria that had not replicated or that had undergone 1 or 2 divisions were analysed.

Importantly, prior to conducting experiments using pFCiGi in mice, *S.* Typhimurium carrying pFCiGi were assessed for correct fluorescence production *in vivo*. Earlier experiments established that arabinose was not present in sufficient levels within the gastrointestinal tract of mice, to induce P_{BAD}-mediated expression of mCherry (Fig. 4.2). Indeed, similar results carried out with uninduced *S.* Typhimurium carrying pFCiGi showed that there was also no significant increase in the level of P_{BAD}-mediated red fluorescence production in these bacteria, compared to the inoculum (Fig. 4.8A). Since P_{lac} can be induced by the natural sugar lactose, as well as the artifical sugar IPTG, the possibility that lactose could be present in sufficient levels induce fluorescence production *in vivo* was tested. As expected, these experiments also showed that there was no significant increase of P_{lac}-mediated green fluorescence production in *S.* Typhimurium in mice (Fig. 4.8B).

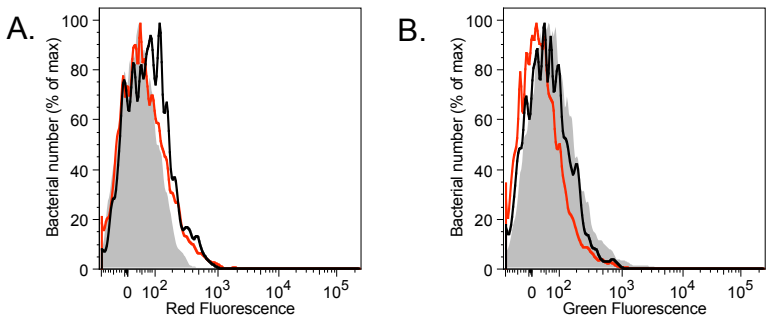


Figure 4.8. Arabinose and IPTG induction of intrabacterial fluorescent proteins in mice. Mice were infected orally with uninduced *S.* Typhimurium carrying pFCiGi and sacrificed after 72 h. Infected tissue homogenates were labelled with primary anti-CSA-1 antibodies and secondary antibodies conjugated to a far-red fluorescent dye, then analysed by flow cytometry. Bacteria from the inoculum (grey), PP (black) and MLN (red) that emitted high levels of far-red fluorescence were subsequently analysed for arabinose-induced red (A) and IPTG-induced green (B) fluorescence production.

4.2.5.2. Fluorescence profiles of mCherry and GFP in *S.* Typhimurium recovered from the SI, PP and MLN

Next, experiments were conducted to compare the fluorescence profiles of mCherry and GFP in mice, to determine if replication-independent loss of bacterial GFP fluorescence occurs *in vivo*. The fluorescence profiles of bacteria recovered from different tissues were examined, as the changing environments at different sites may exert different levels of stress on the bacteria. These investigations were also conducted at early time-points p.i. for two reasons: in order to be accurately compared, the fluorescence intensity of both mCherry and GFP must be above the limit of detection and alongside this, preliminary experiments showed that bacterial replication *in vivo* was often very rapid.

S. Typhimurium carrying pFCiGi was grown for 16 h in MM, with arabinose and IPTG, and subcultured in LB broth to induce SPI-1 T3SS expression. These bacteria were then resuspended in an alkaline buffer (3% $NaHC0_3$ in PBS) at a concentration of 2×10^{10} CFU, to aid neutralisation of gastric acid and increase the numbers of bacteria reaching the intestine and other extraintestinal organs. Mice were inoculated i.g. and after 6 h, animals were sacrificed and the SI, PP and MLN were removed and prepared for flow cytometric analysis. Each sample represents the pooled tissues from 5 mice. Following host tissue homogenisation and cell lysis, samples were labelled with anti-CSA-1 antibody, followed by a secondary labelling with antibody conjugated to a far-red fluorescent dye. Bacteria were identified from autofluorescent host tissue fragments as described above (Section 4.2.5.1.): CSA-1 labelled particles emitting high levels of red fluorescence intensity were considered bacteria. The green fluorescence profile of this high red fluorescent population was then examined for evidence of a population with low green fluorescence intensity. This would represent a replication-independent loss of fluorescence.

In both the SI and PP, there was a proportion of bacteria with high red fluorescence that simultaneously displayed a heterogeneous, low intensity of

green fluorescence (Fig. 4.9A-B). Given that intrabacterial mCherry is more stable than GFP, this provides strong evidence that a significant proportion of the GFP fluorescence loss observed in bacteria recovered from the SI and PP is due to replication-independent factors. The degree of replication-independent green fluorescence loss was quantified by calculating the proportion of bacteria that displayed a level of green fluorescence intensity that was below the level of corresponding red fluorescence intensity. All bacteria that were analysed at each site were those that expressed a level of red fluorescence intensity similar to that of the inoculum (Fig. 4.9Ai, Bi, Ci). Therefore, the amount of GFP instability was quantified by drawing a gate to determine the proportion of bacteria that displayed a green fluorescence intensity lower than that of the inoculum (Fig. 4.9Aii, Bii, Cii). This demonstrated that although the majority of green fluorescence expressed by bacteria in the SI and PP was at an intensity comparable to that of the inoculum, in the SI approximately 40% of bacteria had a green fluorescence intensity lower than that of the inoculum (Table 4.1). In the PP, this proportion was approximately 30% (Table 4.1). However, it is possible that some of the events in the PP represent contamination of bacteria from the SI, as these anatomical sites are part of a continuum and are difficult to completely separate by dissection. In conclusion, the proportion of intrabacterial GFP instability identified in the SI and PP must be taken into account when analysing fluorescence dilution data based on measurement of GFP dilution.

For reasons that are unclear, bacterial seeding of the MLN was very variable and therefore, the data from the MLN represents only one experiment (of a group of 5 mice). However, interestingly, when *S.* Typhimurium was recovered from the MLN, greater than 95% of the intrabacterial GFP fluorescence intensity corresponded to the same level of intensity with respect to the inoculum as mCherry (Table 4.1). This demonstrates that negligible replication-independent loss of GFP fluorescence occurred at this site. Furthermore, this indicates that only bacteria that have avoided or resisted factors present in the gastrointestinal tract that might affect their fluorescence, progress to successfully seed the MLN. Therefore, these experiments also

highlight the use of pFCiGi as a sensor to derive information about bacterial stress within different murine tissues.

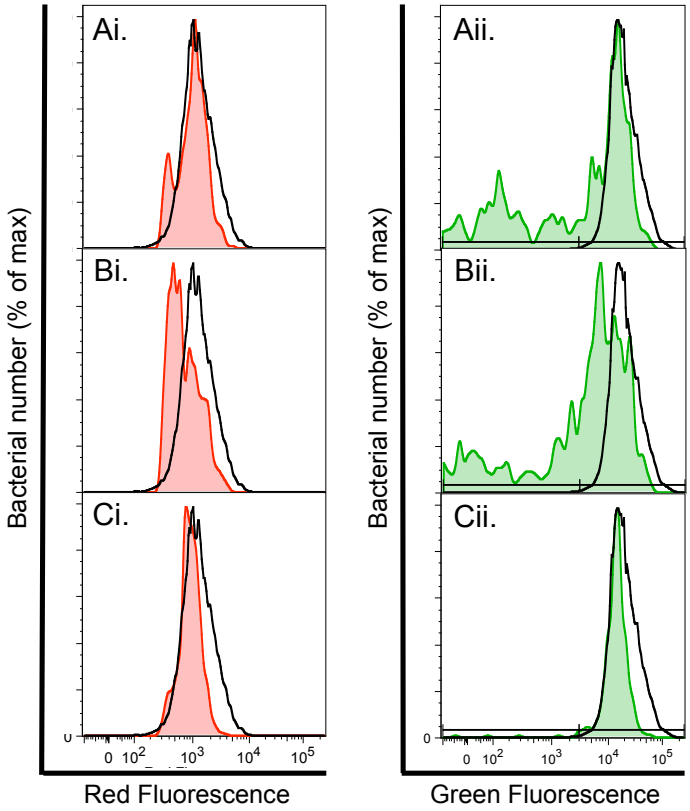


Figure 4.9. Fluorescence profiles of intrabacterial mCherry and GFP in the SI, PP and MLN. Mice were infected orally with pre-induced *S.* Typhimurium carrying pFCiGi. After 6 h, bacteria were recovered from the SI (A), PP (B) and MLN (C) and analsyed by flow cytometry. Bacteria were identified by CSA-1 labelling, followed by gating on high red fluorescence intensity. The level of intrabacterial mCherry fluorescence (red; Ai, Bi, Ci) and GFP fluorescence (green; Aii, Bii, Cii) was compared to that of the inoculum (black). Gates indicate the proportion of bacteria that have a level of GFP intensity higher or lower than that of the inoculum. Data is representative of 1 (C) and 2 (A-B) independent experiments. In each experiment, groups of 5 mice were used.

Body site	Replication-independent GFP fluorescence loss (%)
SI	41 ± 1.9
PP	32 ± 0.7
MLN	2.46

Table 4.1. Percentage replication-independent loss of intrabacterial GFP fluorescence in different body sites in mice. The proportion of bacteria with a greater loss of GFP fluorescence compared to mCherry fluorescence in the SI, PP and MLN.

4.2.5.3. Fluorescence profiles of mCherry and GFP in *S.* Typhimurium recovered from the spleen

Next, experiments were carried out to determine whether there was any replication-independent loss of intrabacterial GFP fluorescence in the spleen. In contrast to the previous experiments, this was conducted using an i.v. route of inoculation, because preliminary experiments revealed that after oral inoculation, *S.* Typhimurium did not reach the spleen in substantial numbers until after approximately 48 h. In addition, these preliminary experiments showed that the majority of bacteria that reached the spleen after oral inoculation had already divided several times, reducing the fluorescence intensity of a dilutable fluorescent protein to levels too low to derive any meaningful measurements. Therefore, i.v. inoculation provided a rapid and direct route to seed the spleen. Mice were inoculated intravenously with 200-1000 CFU of pre-induced dual fluorescent *S.* Typhimurium carrying pFCiGi. Bacteria were prepared in the same manner as described for the experiments above (Section 4.2.5.2.). However, PBS alone was used for final resuspension of the inoculum, as this buffer has a neutral pH, similar to blood. Mice were sacrificed after 1 h and spleens were removed and prepared for flow cytometric analysis. Since preliminary experiments showed that replication was very rapid in the spleen, the red fluorescence of *S.* Typhimurium was too low to use as a means to discard anti-CSA-1 labelling of bacterial debris as outlined earlier (Section 4.2.5.1.). As an alternative method, I used green fluorescence emission to discard non-specific anti-CSA-1 labelling (Fig. 4.10A). Bacteria were clearly identified as a single population with a homogeneous, relatively high level of green fluorescence intensity. There were no particles that displayed intermediate levels of green fluorescence, which would be suggestive of replication-independent green fluorescence loss. To investigate this further, I examined the red fluorescence intensity of these bacteria, to determine if it was similar to their intensity of green fluorescence.

It was first noted that the profiles of fluorescence intensity of both fluorescent proteins were uniform and characteristic of the profiles observed during *S.*

Typhimurium replication in MM (Fig. 3.9B-C). To determine if GFP and mCherry had been diluted to the same extent, the decrease in red and green fluorescence of bacteria was compared to that of the inoculum (Fig. 4.10B-C). By calculating the ratio of the fold decrease of each fluorescent protein, this showed that both fluorescent proteins had diluted at a similar rate. Therefore, this provides strong evidence that there is no replication-independent loss of intrabacterial GFP fluorescence in the spleen.

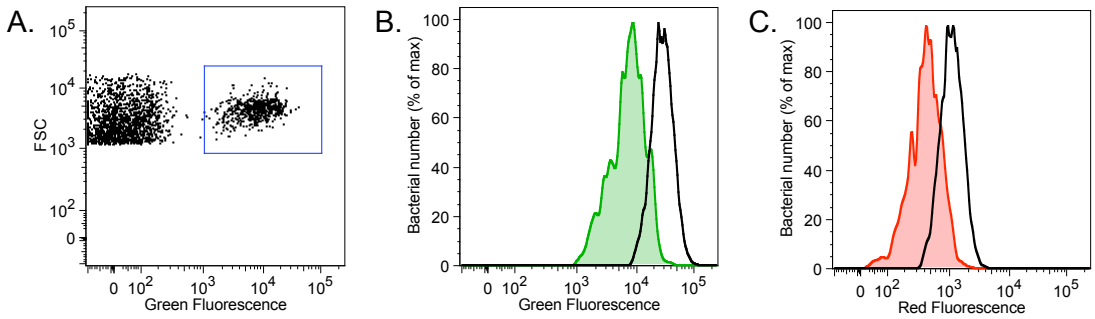


Figure 4.10. Fluorescence dilution profiles of intrabacterial GFP and mCherry in the spleen. Mice were inoculated i.v. with pre-induced *S.* Typhimurium carrying pFCiGi. After 1 h, bacteria were recovered from the spleen and analsyed by flow cytometry. Bacteria were identified by CSA-1 labelling, followed by gating on high green fluorescence intensity. (A) Representative green fluorescence of infected splenic tissue after gating on CSA-1 labelled particles. Gate indicates CSA-1 labelled particles with high green fluorescence that are identified as bacteria. (B-C) Representative profiles of intrabacterial GFP fluorescence (green; B) and mCherry fluorescence (red; C) compared to that of the inoculum (black). Data represents the mean ± SEM for 2 independent experiments. Groups of 5 mice were used in each experiment.

4.3. Summary

In this chapter, I have described experiments to test several variables that might influence the use of GFP fluorescence dilution as a reporter system to measure the replication of *S.* Typhimurium in mice. Importantly, I developed a system to detect whether the fluorescence of a pre-formed pool of GFP in *S.* Typhimurium might be altered by factors other than replication *in vivo*. The results demonstrated that a significant proportion of replication-independent loss of intrabacterial GFP fluorescence occurs in the SI and PP, which is likely to represent exposure of *S.* Typhimurium to acid stress in the gastrointestinal tract. In contrast, no replication-independent loss of GFP fluorescence was observed in the MLN and spleen. Furthermore, since these experiments highlighted the increased sensitivity of intrabacterial GFP to environmental

stress, compared to mCherry, this facilitates the exploitation of pFCiGi as a sensor of bacterial stress *in vivo*. Interestingly, this revealed that only non-stressed bacteria from the SI and/or PP are capable of seeding the MLN.

5. SPATIOTEMPORAL REPLICATION DYNAMICS OF *S.* TYPHIMURIUM DURING SYSTEMIC DISEASE

5.1. Introduction

Until recently, it has been difficult to obtain information about how the replication of *S. enterica* relates to its colonisation of host tissues. In particular, we lack information about the rate and variation of bacterial replication in different tissues and at different stages of the infection, the bacterial virulence determinants that specifically contribute to systemic replication and the host cell factors that influence bacterial replication *in vivo*. Therefore, the overall objective of this study was to gain a better understanding of this, by using fluorescence dilution to examine the replication dynamics of *S.* Typhimurium during systemic disease, in the murine model of acute typhoid fever. In the previous two chapters, I described the development and validation of an optimised dual fluorescence-encoding reporter system, pFCcGi, for use as a tool to measure *S.* Typhimurium replication in mice by fluorescence dilution. Although the experiments showed that intrabacterial mCherry is clearly more stable than GFP when exposed to external stresses, the arabinose-inducible production of mCherry in *S.* Typhimurium was too low to provide a meaningful measure of bacterial replication *in vivo*. Therefore, GFP had to be used as the dilutable marker to measure bacterial replication, as it had a far superior dynamic range of measurement. Importantly, information gained from experiments using pFCiGi enables the identification of profiles that represent replication-independent loss of GFP fluorescence. In this chapter, pFCcGi is used to reveal insights into the extent, rate and population variation of *S.* Typhimurium replication in different tissues during systemic disease.

5.2. Results

5.2.1. Net growth of *S.* Typhimurium in mice following oral inoculation

In the murine model of typhoid fever, orally-acquired *S.* Typhimurium passes along the gastrointestinal tract and gains access to systemic tissues, predominantly by invasion of M cells that overlie the PP in the small intestine (Clark *et al.*, 1994, Jones *et al.*, 1994). Bacteria subsequently colonise the PP, MLN and eventually the liver and spleen. Since *S. enterica* transmission naturally occurs via the oral route, I chose to study the replication dynamics of *S.* Typhimurium in mice after oral inoculation. These investigations were conducted at early stages in the development of disease, as events occurring at this time are crucial to the establishment of a productive infection. The aim was to address specific questions, including: whether *S.* Typhimurium must replicate in order to traverse the intestinal wall and seed systemic organs, whether the replication kinetics of *S.* Typhimurium differs in different tissues and whether all bacteria within one tissue replicate at the same rate. In these experiments, it is important to remember that the colonisation of host tissues by *S.* Typhimurium is a dynamic process governed by several variables, including bacterial replication and killing, dissemination from one organ to another (entry of bacteria to and exit from an individual tissue) and shedding from the host (intestine only).

Mice were inoculated i.g. with 2 x 10^{10} CFU of pre-induced dual fluorescent wild-type *S.* Typhimurium carrying pFCcGi, where GFP acts as a reporter of bacterial replication. Bacterial inocula were prepared as described previously for oral inoculations (see Section 4.2.5.2.). At 2 h, 6 h, 12 h, 24 h and 48 h p.i., mice were sacrificed and bacteria were recovered from the SI, PP, MLN and spleen. Following tissue homogenisation and host cell lysis (except samples recovered from the SI), bacteria were either plated onto LB agar for CFU enumeration or fixed and analysed by flow cytometry. Bacteria were distinguished from autofluorescent host tissue fragments according to size, granularity and red fluorescence intensity (see Section 4.2.1.). The green

fluorescence intensity of these bacteria was then analysed to determine the amount of replication that had taken place.

From 2 h to 48 h after inoculation, the numbers of *S.* Typhimurium in the SI gradually decreased from 5 x 10^7 CFU to 4 x 10^3 CFU (Fig. 5.1A), which is consistent with peristalsis-mediated shedding from the intestine. In the PP, approximately 7 x 10^5 CFU of bacteria were routinely recovered as early as 2 h p.i. (Fig. 5.1A), which supports the finding that *S.* Typhimurium invasion of M cells overlying the PP is a rapid process (Clark *et al.*, 1994, Jones *et al.*, 1994). Following this, the net growth of *S.* Typhimurium in the PP decreased to 4 x 10^4 CFU at 6 h p.i. and remained relatively static from 2 h to 24 h p.i, representing a balance between bacterial influx and/or replication, in conjunction with bacterial killing and/or dissemination to another site. An increase of bacterial numbers in the PP, to over 2 x 10^5 CFU, was then observed at 48 h p.i. In contrast, there was a constant gradual increase in the numbers of *S.* Typhimurium in the MLN over the same time period, from <10 CFU at 2 h to 3 x 10^4 CFU at 48 h p.i. (Fig. 5.1A), suggestive of a low level of seeding and/or bacterial replication in this tissue. Additionally, the total CFU count of *S.* Typhimurium was consistently lower in the MLN than in the PP at all time-points (Fig. 5.1A). In the spleen, very few bacteria (20 CFU or less) were detected from 2 h to 24 h p.i. and only at 48 h p.i. were larger numbers (approximately 500 CFU) of bacteria detected (Fig. 5.1A). The net growth of *S.* Typhimurium in this experiment was calculated per mouse and compared with other published work, bearing in mind that many variables can affect the course of infection, for example: the size of the inoculum and genetics of both the host and bacterial strain. The trend of bacterial growth and the numbers of bacteria recovered from different tissues obtained in these experiments were generally similar to those described in previous reports (Hohmann *et al.*, 1978, Cirillo *et al.*, 1998, Hopkins *et al.*, 2000, Cheminay *et al.*, 2004, McSorley *et al.*, 2002, Salazar-Gonzalez *et al.*, 2006, Voedisch *et al.*, 2009).

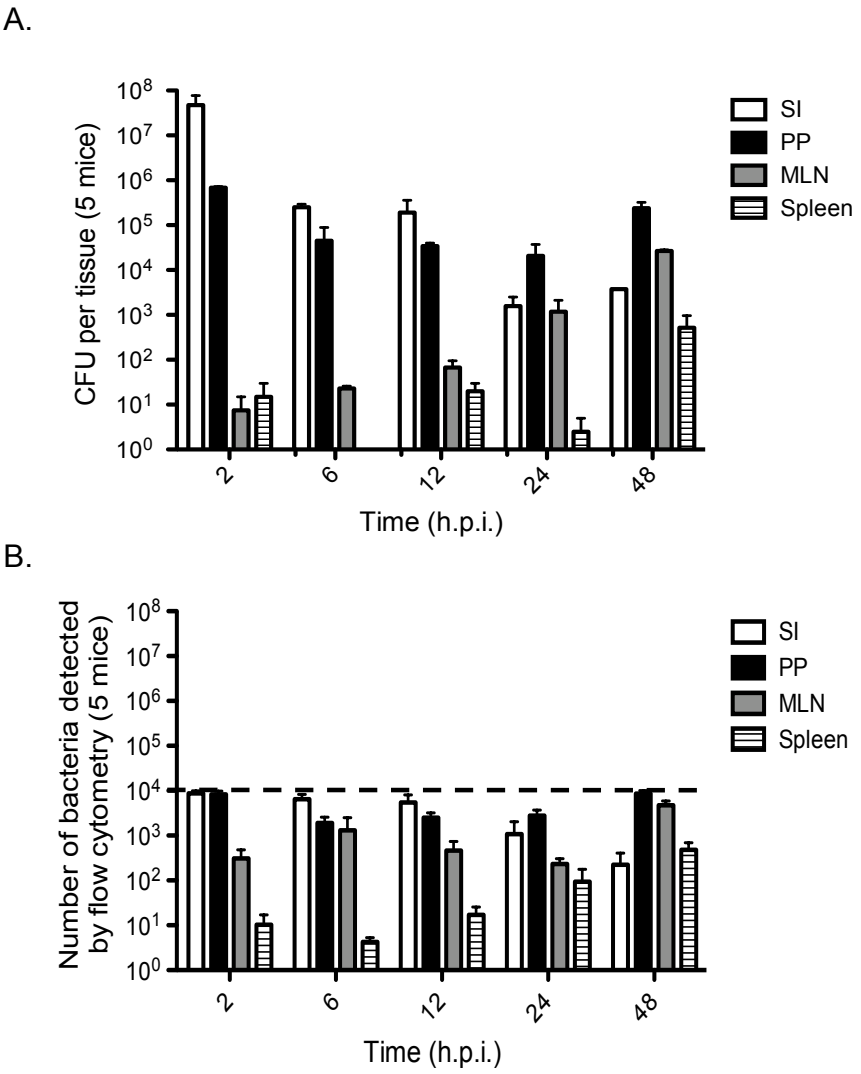


Figure 5.1. Detection of bacteria in different host tissues by CFU and flow cytometry. Mice were inoculated orally with 2 x 10^{10} CFU of pre-induced *S.* Typhimurium carrying pFCcGi. At designated times p.i., mice were sacrificed and bacteria were recovered from the SI, PP, MLN and spleen. The numbers of bacteria at each site were determined by growth on LB agar (A) and by flow cytometric detection of constitutive red fluorescence (B). Dashed line in (B) indicates the maximum number of events acquired (*n* = 0-10,000 bacteria). Groups of 5 mice were used in each experiment. Data represent the mean ± SEM for 2 (A) or 3 to 6 (B) independent experiments.

In separate experiments, the tissues from infected mice were processed for flow cytometric analysis. The number of bacteria detected in each tissue in these experiments was then compared to the number of bacteria recovered by CFU in the previous experiment. However, as acquisition by flow cytometry was terminated when 10 000 events were acquired, comparisons can only be made for the MLN and spleen samples (in which less than 10 000 events were obtained). In the spleen, similar numbers of bacteria were detected by

flow cytometry and CFU, apart from at 24 h p.i., when more bacteria were detected by flow cytometry (Fig. 5.1A-B). From 2 h to 12 h p.i. in the MLN, more bacteria were also detected by flow cytometry, compared to CFU (Fig 5.1A-B) and this did not resemble the gradual increase in net growth that was observed by CFU. One explanation for the detection of more bacteria by flow cytometry compared to CFU, is that the bacteria detected by flow cytometry were non-culturable, perhaps because they were damaged by the host or had entered a dormant state. Other authors described the development of a relapsing infection in mice infected with *S.* Typhimurium, despite the fact that bacteria were no longer culturable by plating from any intestinal or systemic tissues (Griffin *et al.*, 2011). In addition, bacteria that had not replicated, but remained viable, were observed to persist for several days in splenocytes recovered from mice infected with *S.* Typhimurium (Helaine *et al.*, 2010) and a significant proportion of these bacteria were non-culturable (Dr. S. Helaine, personal communication). Furthermore, experiments in the current study suggested that a large proportion of the bacteria detected in the MLN at early time-points had not replicated (see below).

5.2.2. Replication dynamics of *S.* Typhimurium in the SI, PP, MLN and spleen

In experiments where the tissues of infected mice were subjected to flow cytometric analysis, the green fluorescence intensity of *S.* Typhimurium was analysed to determine the profile of bacterial replication. Representative fluorescence dilution profiles of bacteria recovered from different tissues, at different times p.i. and from different experiments are shown in Figure 5.2 and 5.3. Detailed quantification of the fluorescence dilution data from the experiments are shown in Tables 5.1-3.

5.2.2.1. Identification of replication-independent loss of green fluorescence

Experiments described in Chapter 4 revealed that environmental stress can give rise to replication-independent loss of intrabacterial green fluorescence.

Therefore, it was important to identify these events before any conclusions could be made about the replication of *S.* Typhimurium.

The intestinal lumen represents the main site in which *S.* Typhimurium is likely to encounter environmental stress that could cause intrabacterial GFP instability. Indeed, earlier experiments revealed that the highest degree of replication-independent green fluorescence loss was observed in the SI (Table. 4.1). Therefore, no conclusions were made about *S.* Typhimurium replication at this site.

Earlier experiments also demonstrated that a large proportion of bacteria recovered from murine PP displayed evidence of a replication-independent loss of green fluorescence, at least after 6 h post-i.g. inoculation (Table. 4.1). Indeed, close examination of the fluorescence profiles of *S.* Typhimurium recovered from the PP from 2 h to 12 h p.i. revealed populations of bacteria that displayed heterogeneous, low levels of green fluorescence intensity ('black circles' in Fig. 5.2). These profiles are characteristic of the replication-independent loss of green fluorescence described in experiments conducted in Chapter 4, using pFCiGi (Fig. 4.9Aii, Bii). Therefore, these populations were not considered to represent *S.* Typhimurium replication and were not included in the analysis in Tables 5.2 and 5.3. On the other hand, the fluorescence profiles of *S.* Typhimurium recovered from the PP also clearly revealed a population of bacteria that displayed normally distributed, intermediate levels of green fluorescence (blue, Fig. 5.2). Since these profiles are characteristic of bacterial replication (Fig. 3.8C), they were included in the replication analysis, illustrated in Tables 5.2 and 5.3.

Since earlier experiments revealed no evidence of replication-independent intrabacterial GFP fluorescence loss in the MLN at 6 h post-i.g. inoculation (Table 4.1) and in the spleen at 1 h post-i.v. inoculation (Fig. 4.10) of mice, all low green fluorescent populations of bacteria recovered from these tissues, at all time-points, were considered to have undergone replication. The number of divisions undergone by these bacteria was quantified and is illustrated in Tables 5.2 and 5.3.

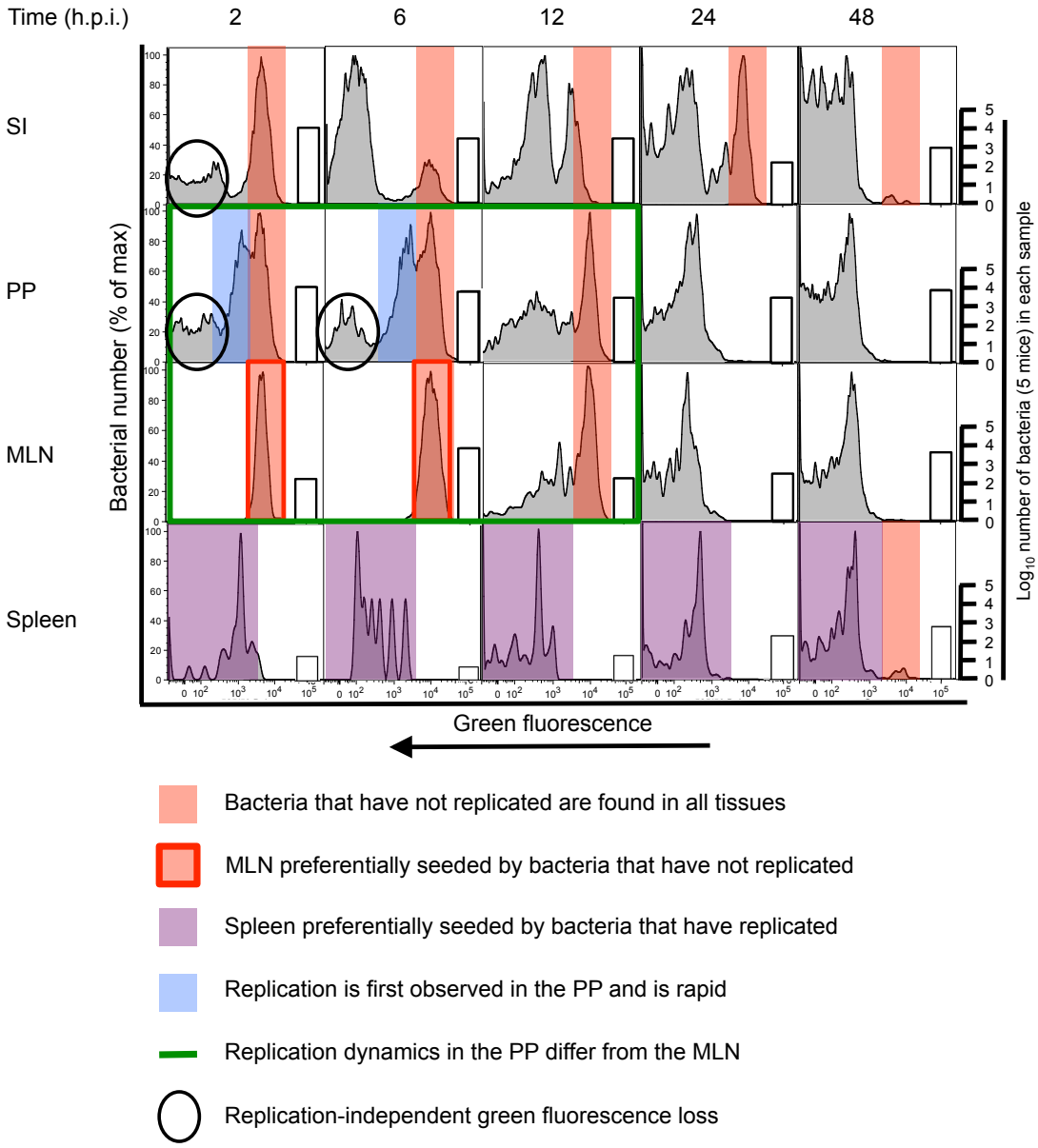


Figure 5.2. Replication dynamics of *S.* Typhimurium in different host tissues during a systemic infection. Mice were inoculated i.g. with pre-induced wild-type *S.* Typhimurium carrying pFCcGi. At designated time-points, bacteria were recovered from the SI, PP, MLN and spleen and green fluorescence was analysed by flow cytometry. The data illustrate representative green fluorescence dilution profiles of *S.* Typhimurium in different tissues, taken from different experiments. Although a representative profile for the spleen at 6 h is shown, this represents <20 events and is not quantified in Table 5.2. White bars represent the number of bacteria detected in each sample, by flow cytometry. Groups of 5 mice were used in each experiment. Data are representative of 3 to 6 independent experiments.

Time (h.p.i.)	Experiment	SI			PP			MLN			Spleen		
		NR (%)			NR (%)			NR (%)			NR (%)		
		Individual	Average	SEM	Individual	Average	SEM	Individual	Average	SEM	Individual	Average	SEM
2	1	99	70	15.32	82	50	11.69	93	95	1.35	-	-	-
	2	85			47			94			-		
	3	81			31			-			3		
	4	11			20			94			-		
	5	75			71			99			-		
6	1	81	89	3.77	51	52	11.07	91	64	19.47	-	-	-
	2	83			70			99			-		
	3	95			62			15			-		
	4	95			85			-			-		
	5	-			8			50			-		
	6	-			37			-			-		
12	1	-	21	4.37	37	24	4.02	53	43	15.12	83	-	-
	2	12			26			97			-		
	3	24			26			23			-		
	4	-			13			29			0		
	5	26			19			12			-		
24	1	54	42	10.32	0	2	2.00	38	9	7.30	-	-	-
	2	25			0			1			-		
	3	64			10			5			-		
	4	23			0			0			-		
	5	-			0			1			0		
48	1	1	2	0.50	0	0	0.00	0	0	0.33	4	2	1.20
	2	-			0			0			1		
	3	-			0			1			0		
	4	2			ND			ND			ND		

Table 5.1. Proportion of non-replicating bacteria in the SI, PP, MLN and spleen. The proportion of non-replicating (NR) bacteria in the SI, PP and MLN at designated times following oral inoculation of mice. Shaded values represent experiments where the proportion of NR bacteria is greater in the MLN than in the PP. Groups of 5 mice were used in each experiment. ND (Not done). (-) indicates the exclusion of samples that represent ≤ 20 bacteria.

Time (h.p.i.)	Experiment	PP			MLN			Spleen		
		Replicating bacteria (number of generations)			Replicating bacteria (number of generations)			Replicating bacteria (number of generations)		
		Individual	Average	SEM	Individual	Average	SEM	Individual	Average	SEM
2	1	2.8	2.7	0.23	0.0	0.0	0.00	-	-	-
	2	2.5			0.0			-		
	3	2.3			-			3.4		
	4	3.6			0.0			-		
	5	2.4			0.0			-		
6	1	3.4	3.0	0.24	2.4	2.5	0.93	-	-	-
	2	2.1			0.0			-		
	3	2.9			3.4			-		
	4	2.8			-			-		
	5	3.8			4.3			-		
	6	2.9			-			-		
12	1	5.2	5.3	0.27	4.3	3.2	0.82	3.0	3.9	0.85
	2	6.2			0.0			-		
	3	5.4			4.6			-		
	4	5.3			3.4			4.7		
	5	4.5			3.5			-		
24	1	5.8	6.0	0.09	5.5	5.8	0.26	-	-	-
	2	6.0			5.2			-		
	3	5.8			5.5			-		
	4	6.3			6.7			-		
	5	6.1			6.1			4.0		
48	1	6.8	6.1	0.34	5.9	5.9	0.10	5.0	4.9	0.46
	2	5.9			6.1			4.1		
	3	5.7			5.7			5.7		
	4	ND			ND			ND		

Table 5.2. Average bacterial replication in the PP, MLN and spleen. The average number of divisions undergone by bacteria in the PP and MLN at designated times following inoculation. Shaded values represent experiments where bacteria in the MLN have replicated less than in the PP. Groups of 5 mice were used in each experiment. ND (Not done). (-) indicates the exclusion of samples that represent ≤ 20 bacteria.

Since exposure to environmental stress resulted in a loss of green fluorescence intensity, this suggests that bacteria retaining high levels of green fluorescence intensity, comparable to bacteria in the inoculum, had not been affected by environmental stress. Retention of high levels of green fluorescence intensity also shows that these bacteria have not replicated. Therefore, conclusions could be made about these bacteria, regardless of the tissue in which they had been recovered from. Quantification of the proportions of bacteria that had not replicated (also referred to as non-replicating bacteria) recovered from the SI, PP, MLN and spleen are illustrated in Table 5.1.

5.2.2.2. Analysis of bacterial replication by fluorescence dilution

In contrast to cell culture systems, a large amount of inter-experiment variation is expected when using animal models, since more variables exist and these cannot all be precisely controlled. However, despite this variation, several consistent observations could be made about the replication dynamics of *S.* Typhimurium within the SI, PP, MLN and spleen.

Bacteria that had not replicated were found in all sites at different times throughout the infection (Fig. 5.2). These were quantified, shown in Table 5.1. These results reveal that replication is not a prerequisite for *S.* Typhimurium to traverse the intestinal wall and enter the PP, or to seed the MLN and the spleen.

In the SI, large proportions (ranging from approximately 20% to 90%) of non-replicating bacteria were detected from 2 h to 24 h p.i. (Table 5.1). Non-replicating bacteria were still detected at 48 h p.i., however at this time-point much smaller proportions (approximately 2%) were found (Table 5.1). This suggests that non-replicating bacteria are able to persist within the intestinal lumen for at least 48 h.

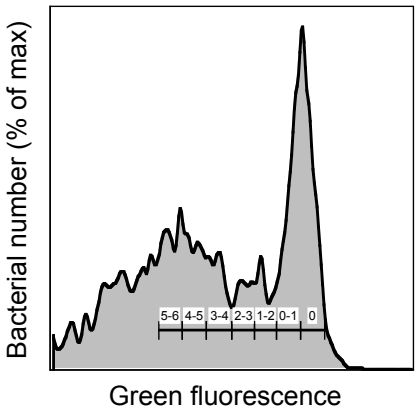


Figure 5.3. Variation of bacterial replication *in vivo*. Representative green fluorescence dilution profile of pre-induced wild-type *S.* Typhimurium carrying pFCcGi recovered from the PP at 12 hfollowing i.g. inoculation of mice. Gates indicate the number of divisions undergone by bacteria.

Time (h.p.i.)	Replication (number of generations)	Proportion of bacteria (%)		
		PP	MLN	Spleen
2	0	7	29	-
	0-1	27	50	-
	1-2	22	14	-
	2-3	23	2	-
	3-4	16	0	-
	4-5	5	0	-
	5-6	0	0	-
12	0	17	21	0
	0-1	15	23	0
	1-2	6	11	0
	2-3	6	8	0
	3-4	8	11	11
	4-5	10	9	26
	5-6	11	6	6
48	0	0	0	1
	0-1	0	0	2
	1-2	0	0	0
	2-3	1	1	1
	3-4	2	4	3
	4-5	15	24	26
	5-6	17	20	19

Table 5.3. Spectrum of bacterial replication within the PP, MLN and spleen. The proportion of bacteria, from representative experiments, that have undergone the designated number of divisions within the PP, MLN and spleen at different times post-i.g. inoculation of mice with pre-induced wild-type *S.* Typhimurium carrying pFCcGi. Groups of 5 mice were used in each experiment.

Surprisingly, at 2 h p.i. the MLN were preferentially seeded by bacteria that had not replicated, despite the presence of replicating bacteria in the PP (Fig. 5.2, Tables 5.1 and 5.2). Earlier experiments with pFCiGi strongly suggest that these bacteria have avoided or resisted environmental stress in the gastrointestinal tract (Table 4.1). Replicating bacteria were subsequently found in the MLN at later time-points, from 6 h to 48 h p.i., and this was corroborated by the increase in net growth, measured by CFU (Fig. 5.1A).

When non-replicating bacteria were observed in the MLN, replication had already begun in the PP (Fig. 5.2). This was relatively rapid, as approximately 45% of bacteria had already undergone 1 to 3 divisions within 2 h of infection (Table 3). It is possible that some of the bacteria recovered in the PP sample were located on the luminal side of the PP tissue and therefore, represent contaminating bacteria from the SI. However, the replicating population of bacteria observed in the PP at 2 h was never observed in the SI, providing strong evidence that this replication only took place once *S.* Typhimurium had entered the PP. Bacterial replication in the PP then continued steadily from 2 h to 48 h p.i. (Fig. 5.2). Interestingly, the replication observed by fluorescence dilution in the PP from 2 h to 24 h p.i. was not accompanied by an increase in net growth by CFU (Fig. 5.1A). However, many factors are likely to affect the numbers of viable bacteria recovered from this site, including: potential host cell killing of bacteria and/or dissemination of bacteria to other tissues. Eventually this replication did lead to an increase in bacterial net growth, from 24 h to 48 h p.i. (Fig. 5.1A).

Bacteria that had not replicated were found in both the PP and the MLN at all time-points from 2 h to 24 h p.i. (Table 5.1). The overall proportion of these bacteria gradually declined in both tissues as the infection progressed and as the replicating population increased (Fig. 5.4A-B, Tables 5.1 and 5.2). At 48 h p.i., non-replicating bacteria were no longer detected (Fig. 5.4A and Table 5.1). It is possible that these bacteria eventually begin to replicate or are killed by the host. Alternatively, non-replicating bacteria may simply become too difficult to detect, since they represent an increasingly small proportion of the total population of bacteria within the tissue, given the increasing numbers of

replicating bacteria that accumulate over time. Despite this decline, the MLN consistently contained a higher average proportion of bacteria that had not replicated, compared to the PP (Fig. 5.4A and Table 5.1). At 2 h p.i., significantly more ($p < 0.01$) non-replicating bacteria were found in the MLN compared to the PP (Fig. 5.4A). At the same time, the average number of divisions undergone by bacteria in the MLN was consistently lower than in the PP (Fig. 5.4B). This difference was significant at 2 h ($p < 0.001$) and 12 h p.i. ($p < 0.05$). The average replication of bacteria in the MLN was also slightly lower than in the PP at 24 h and 48 h p.i. (Table 5.2). However, it is likely that this represents an underestimation in both cases, as the limit of GFP sensitivity is reached after approximately 6 generations of replication. In conclusion, there were more non-replicating bacteria and less replication of bacteria in the MLN compared to the PP, which suggests that the MLN represent a more restrictive site for *S.* Typhimurium replication compared to the PP.

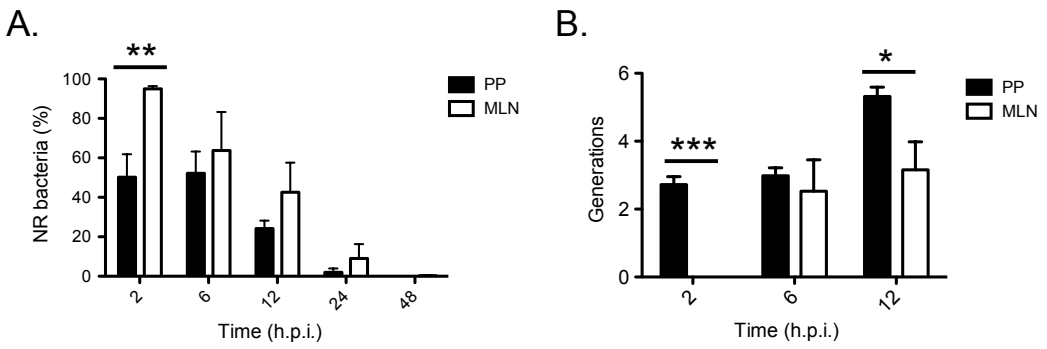


Figure 5.4. Bacterial replication dynamics in the PP and MLN. Mice were inoculated orally with pre-induced wild-type *S.* Typhimurium carrying pFCcGi. Bacteria were recovered from the PP and MLN at the designated times p.i. and the degree of bacterial replication was determined by analysis of green fluorescence dilution using flow cytometry. (A) Proportion of non-replicating (NR) bacteria and (B) average replication of bacteria within the PP and MLN. Groups of 5 mice were used in each experiment. Data represents the mean ± SEM for 3 to 6 independent experiments. Data was analysed using a non-paired one-tailed student's *t* test and values that were significantly different are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Whereas large proportions of non-replicating bacteria were frequently found in the SI, PP and MLN (Table 5.1), in the spleen, only small proportions of non-replicating bacteria were detected in most experiments at all time-points. However, in one experiment, 83% of bacteria recovered from the spleen were non-replicating (Table 5.1). Instead, the majority of bacteria first detected in

the spleen had already replicated (Fig. 5.2 and Table 5.2), for example 43% and 48% of bacteria had already undergone 3 to 6 divisions at 12 h and 48 h p.i., respectively (Table 5.3). This indicates that *S.* Typhimurium replicated in other tissues, such as the SI, PP or MLN, prior to reaching the spleen.

The fluorescence dilution profiles of *S.* Typhimurium recovered from different murine tissues indicated that there was a great deal of variation in the extent of bacterial replication within an individual site. At each time point, the total population of bacteria detected in the PP, MLN and spleen consisted of different proportions of bacteria that had undergone 0 through to 6 or more divisions (Fig. 5.3 and Table 5.3). For example, at 48 h p.i., 3%, 4% and 45% of bacteria in the spleen had undergone 0 to 1, 2 to 4 and 4 to 6 divisions, respectively. Heterogeneous profiles such as these could be the result of a number of variables, including differences in the initiation of replication, differences in replication rate and possibly, dissemination of bacteria from one organ to another.

5.2.3. Bacterial replication within different host cell types in the PP and MLN

It is possible that the replication of *S.* Typhimurium within the PP and MLN might be host cell type dependent, since *S.* Typhimurium has been found to replicate at different rates within different host cell types, such as macrophages and DCs (Schwan *et al.*, 2000, Jantsch *et al.*, 2003).

To investigate relationships between host cell type and bacterial replication, mice were inoculated orally as above, with pre-induced wild-type *S.* Typhimurium carrying pFCcGi, and at 12 h p.i. mice were sacrificed and the PP and MLN were removed. This time-point was chosen, as both replicating and non-replicating bacteria were routinely recovered from the PP and MLN at this time (Tables 5.1 and 5.2). To increase the sample size, tissues from 10 mice were pooled together. Cell suspensions of infected tissues were generated and these were separated into $CD11b^+$ and $CD11c^+$ cell fractions using magnetic bead cell separation. CD11b or integrin alpha M is a subunit

of the macrophage-1 antigen, also known as complement receptor (CR)3. It is highly expressed on monocytes and macrophages and to a lesser extent on granulocytes and certain DCs (Springer *et al.*, 1979, Ho and Springer, 1982, Liu and Nussenzweig, 2010). CD11c or integrin alpha X is a subunit of CR4 and is a widely used marker of DCs, although it can be expressed to a lower extent on activated macrophages (Metlay *et al.*, 1990). These cell fractions were subsequently lysed, to release intracellular bacteria, and bacterial fluorescence was detected by flow cytometry. The unlabelled cell fraction, which also included extracellular bacteria, was treated in the same way. In this manner, the replication of *S.* Typhimurium within broadly different host cell types in the PP and MLN could be analysed.

In most experiments, bacteria that had not undergone any replication were detected in all cell fractions in the PP and MLN (Fig. 5.5A-B and Table 5.4). On average, a higher proportion (approximately 62%) of these non-replicating bacteria were detected in the MLN, compared to the PP (approximately 46%) (Fig. 5.5C), which is in agreement with earlier observations (Fig. 5.4A and Table 5.1). However, there was no significant difference in the proportions of non-replicating bacteria detected within the different host cell fractions (Fig. 5.5C and Table 5.4). In addition to bacteria that had not replicated, those that had replicated were also detected in all cell fractions in the PP and MLN, in most experiments (Fig. 5.5A-B and Table 5.5). The average replication of *S.* Typhimurium in the MLN was lower (approximately 3.6 generations) compared to in the PP (approximately 4.6 generations) (Fig. 5.5D), which is in agreement with earlier experiments (Fig. 5.4B and Table 5.2). However, this was not statistically significant. In addition, there was no significant difference between the amount of bacterial replication that took place within certain host cell types in the PP compared to the MLN (Fig. 5.5D and Table 5.5).

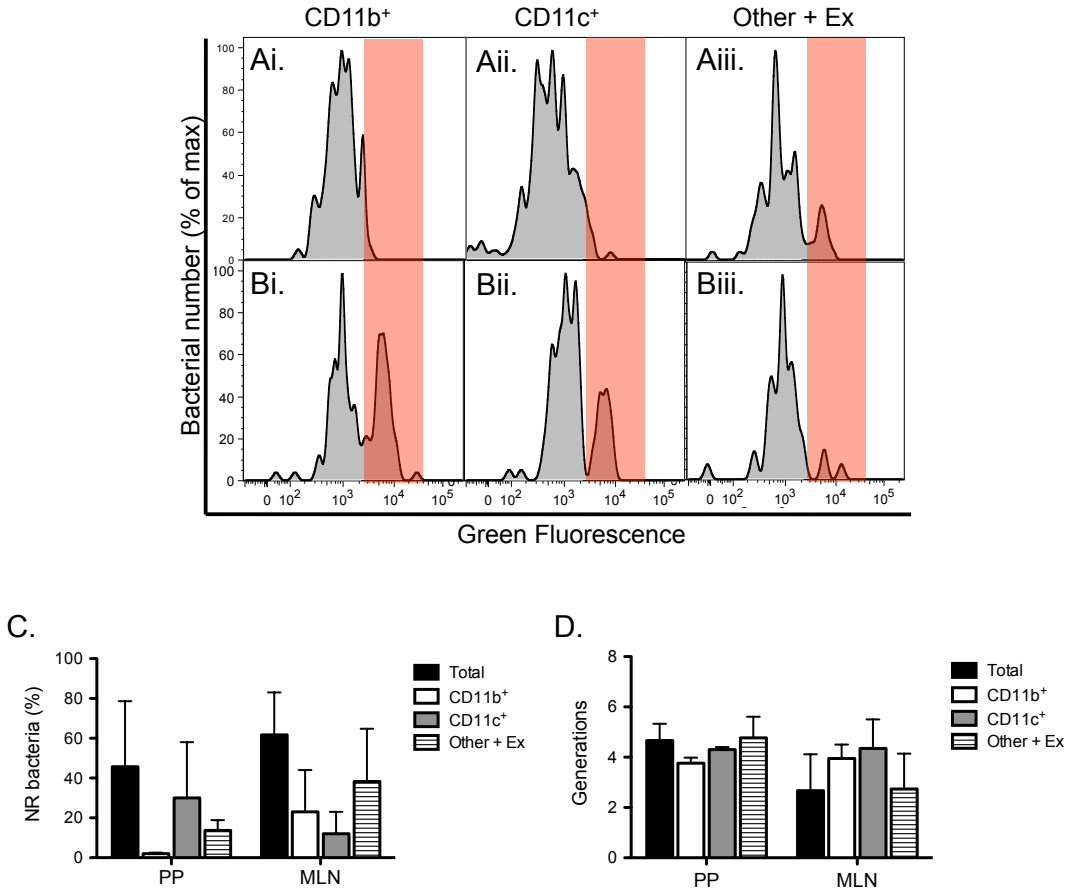


Figure 5.5. Bacterial replication within different host cell types in the PP and MLN. Flow cytometric detection of green fluorescence expression in pre-induced wild-type *S.* Typhimurium carrying pFCcGi after oral inoculation of mice. At 12 h.p.i., the PP (A) and MLN (B) were removed and homogenised. Cell suspensions were separated into a CD11b$^+$ (Ai, Bi), CD11c$^+$ (Aii, Bii) and unlabelled cell fraction that also contained extracellular bacteria (Aiii, Biii). These were then lysed to release intracellular bacteria. Bacterial GFP dilution was analysed using flow cytometry to determine the extent of bacterial replication. Groups of 10 mice were used in each experiment. Data is representative of 3 independent experiments. (C-D) Proportion of non-replicating (NR) bacteria and average replication of bacteria within all samples (total) or designated cell fractions. Data represents the mean ± SEM for 3 independent experiments. Unlabelled cell fraction and extracellular bacteria (Other + Ex).

Time (h.p.i.)	Experiment	PP			MLN		
		NR (%)			NR (%)		
		CD11b$^+$	CD11c$^+$	Other + Ex	CD11b$^+$	CD11c$^+$	Other + Ex
12	1	2	4	15	44	23	7
	2	1	0	4	2	1	17
	3	3	86	22	-	-	91

Table 5.4. Proportion of non-replicating bacteria within different host cell types in the PP and MLN. The proportion of non-replicating (NR) wild-type *S.* Typhimurium carrying pFCcGi detected within CD11b$^+$ cells, CD11c$^+$ cells and unlabelled cells plus extracellular bacteria (Other + Ex) in the PP and MLN at 12 h.p.i. Groups of 10 mice were used in each experiment.

Time (h.p.i.)	Experiment	PP			MLN		
		Replicaton (number of generations)			Replicaton (number of generations)		
		CD11b$^+$	CD11c$^+$	Other + Ex	CD11b$^+$	CD11c$^+$	Other + Ex
12	1	3.5	4.2	3.6	3.4	3.2	3.5
	2	4.5	4.4	4.3	4.5	5.5	4.7
	3	5.7	-	6.4	-	-	0.0

Table 5.5. Replication of bacteria within different host cell types in the PP and MLN. The average extent of replication undergone by wild-type *S.* Typhimurium carrying pFCcGi within CD11b$^+$ cells, CD11c$^+$ cells and unlabelled cells plus extracellular bacteria (Other + Ex) in the PP and MLN at 12 h.p.i. Groups of 10 mice were used in each experiment.

Therefore, these experiments suggest that the differential replication of *S.* Typhimurium within the MLN and PP is not related to the host cell type. However, it is difficult to draw firm conclusions from these data, as there was a large degree of variation between experiments. Small numbers of bacteria (approximately 100 bacteria per cell fraction) were recovered from the PP and MLN in these experiments, compared to earlier experiments (Fig 5.1B), which was most likely due to the more gentle tissue homogenisation required to obtain intact cell suspensions. As a result, these small samples may be less representative of the entire intracellular population of bacteria and therefore, may have generated more variation. In addition, it is possible that the separation of different host cell types was not efficient, since neither CD11b or CD11c are exclusive to a particular host cell type. Nevertheless, it is interesting to note that in most experiments, bacteria that had undergone between 3 and 6 divisions were also found within CD11c$^+$ cells (Table 5.5), suggesting that *S.* Typhimurium could have replicated within DCs. This was unexpected, since *S.* Typhimurium is not thought to replicate efficiently within DCs (Niedergang *et al.*, 2000, Jantsch et al., 2003, Bueno *et al.*, 2008). However, it is possible that these cells could have phagocytosed bacteria after they replicated extracellularly or within another cell type.

5.2.4. Contribution of the SPI-2 T3SS to replication of *S.* Typhimurium *in vivo*

In addition to investigating the potential influence of host cell type on *S.* Typhimurium replication *in vivo*, I also wanted to determine the impact of bacterial factors on *S.* Typhimurium replication in the mouse model. The SPI-

2 T3SS has been shown to facilitate the intracellular growth of *S.* Typhimurium within host cells (Ochman *et al.*, 1996, Hensel *et al.*, 1998) and to be essential for efficient colonisation of the MLN, liver and spleen of mice (Hensel *et al.*, 1995, Cirillo *et al.*, 1998, Shea *et al.*, 1999). Therefore, fluorescence dilution was used to further clarify the contribution of the SPI-2 T3SS to *S.* Typhimurium replication *in vivo*.

Mice were inoculated intravenously as described earlier (see Section 4.2.5.3.) with a low dose (200-1000 CFU) of pre-induced wild-type or SPI-2 T3SS mutant *S.* Typhimurium carrying pFCcGi. This mutant contains a deletion in *ssa*V (*Δssa*V), which encodes a protein required for the assembly of the SPI-2 T3SS (Beuzon *et al.*, 2000). As a result, no SPI-2 effectors are translocated by this strain. An i.v. route of inoculation was chosen to facilitate direct seeding of the spleen, as earlier experiments revealed that the majority of bacteria first detected in the spleen, after oral inoculation, had already undergone several divisions (Fig. 5.2), thereby reducing the measurable range of GFP dilution at this site. However, of course the disadvantage of this is that it represents an artificial route of inoculation.

Groups of 8 infected mice were sacrificed at 2 h, 6 h and 8 h p.i. and their spleens were removed and homogenised together in a cell lysis buffer, to release intracellular bacteria. Early time-points were investigated, as preliminary experiments revealed that the replication of *S.* Typhimurium in the spleen was very rapid and the limit of GFP dilution was reached well within 24 h of infection. A fraction of the homogenate was then removed and fixed for flow cytometric analysis, while another fraction was plated for CFU enumeration. Since fluorescence dilution provides a direct measure of bacterial replication and CFU provides a measure of net growth, the difference between these two parameters can provide a measure of the amount of bacterial killing, termed the ‘killing index’ (Helaine *et al.*, 2010). When measuring bacterial growth dynamics within the host, it is important to bear in mind that the population of bacteria in a particular tissue is not only influenced by replication and killing, but also by spread of bacteria between different sites. However, work described in another report indicates that

bacterial spreading between different organs at the time-points investigated in this study would be very unlikely (Grant *et al.*, 2008).

Fluorescence dilution revealed that the replication of both wild-type and *Δssa*V strains was relatively homogeneous in the spleen (Fig. 5.6A-B), which contrasts with the relatively heterogeneous replication of *S.* Typhimurium observed in different tissues after oral inoculation of mice (Fig. 5.3). Instead, the normally distributed profiles of bacterial replication in the spleen were reminiscent of the replication profiles of *S.* Typhimurium in MM *in vitro* (Fig. 3.8C). The total population of bacteria recovered at each time-point consisted of different proportions of bacteria that had undergone varying numbers of divisions (Fig. 5.6A-B). For example, at 8 h p.i., 1%, 19%, 40% and 39% of wild-type and 10%, 34%, 44% and 10% of *ΔssaV* bacteria had undergone 0 to 2, 2 to 4, 4 to 6 or 6+ divisions, respectively (Table 5.6). Surprisingly, by 8 h p.i., all bacteria of both strains had undergone replication (Fig. 5.6A-B and Table 5.6), which is in contrast with the identification of non-replicating bacteria in different host tissues, including the SI, PP, MLN and spleen following oral (Fig. 5.2 and Table 5.1) or i.p. (Helaine *et al.*, 2010) inoculation of mice with wild-type *S.* Typhimurium.

During the first 2 h, wild-type *S.* Typhimurium replicated at a very rapid rate, dividing every 0.7 h (1.2 generations per h) (Fig 5.6C). This was accompanied by a slightly higher rate of killing (1.4 generations per h) (Fig. 5.6D). However, this most likely represents an over-estimation of the killing rate, since not all bacteria in the inoculum will seed the spleen. Over the next 6 h, the rate of replication gradually slowed to approximately 2.8 h per division (0.35 generations per h) and by 8 h p.i. the majority of bacteria had undergone over 4 divisions (Fig. 5.6C and Table 5.6). This was paralleled by a significant decrease in the killing rate to negligible levels by 8 h p.i. (0.1 generations per h) (Fig. 5.6D). These results are consistent with the dynamics of bacterial growth described in other work, using WITS to provide a proxy measure of bacterial replication and killing in the spleen following i.v. infection of mice (Grant *et al.*, 2008).

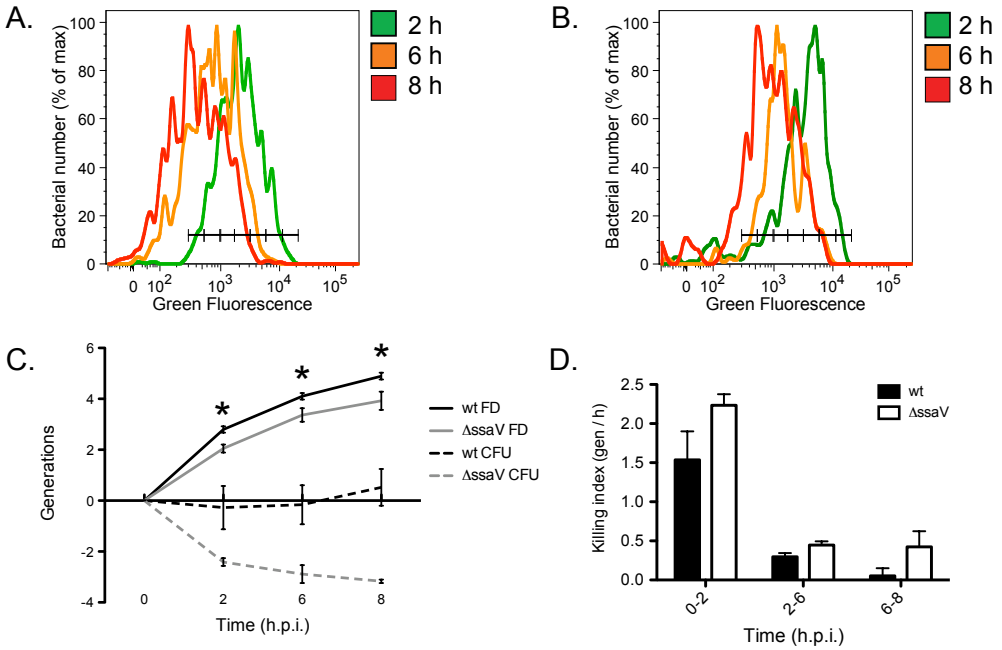


Figure 5.6. Contribution of SPI-2 to replication *in vivo*. Mice were inoculated intravenously with 200-1000 CFU of pre-induced wild-type and *Δssa*V *S.* Typhimurium carrying pFCcGi. At 2 h, 6 h, and 8 h p.i., bacteria were recovered from the spleens and analysed by flow cytometry or plated for CFU enumeration. (A-B) Representative profiles of green fluorescence expression in wild-type (A) and *Δssa*V (B) bacteria. Gates indicate number of divisions undergone by bacteria, as follows: 0, 0-1, 1-2, 2-3, 3-4, 4-5, 5-6 from right to left. (C) Replication (fluorescence dilution, FD) and net growth (CFU) of wild-type and *Δssa*V bacteria. (D) Killing rate of wild-type and *Δssa*V bacteria. Groups of 8 mice were used in each experiment. Data represents the mean ± SEM for 3 independent experiments. Data was analysed using a paired, one-tailed student's *t* test and values that were significantly different ($p < 0.05$) are indicated with an asterisk (*).

Time (h.p.i.)	Replication (number of generations)	Proportion of bacteria (%)	
		wild-type	Δ*ssa*V
8	0	0	0
	0-1	0	2
	1-2	1	8
	2-3	7	14
	3-4	12	20
	4-5	18	25
	5-6	22	19
	6+	39	10

Table 5.6. Spectrum of replication undergone by wild-type and *Δssa*V *S.* Typhimurium in the spleen. Mice were inoculated intravenously with 200-1000 CFU of pre-induced wild-type or *Δssa*V *S.* Typhimurium carrying pFCcGi. The proportion of bacteria in the spleen at 8 h p.i. that have undergone the designated number of divisions is shown.

In comparison to wild-type bacteria, the replication of the SPI-2 T3SS-deficient strain was significantly less at all time-points ($p < 0.05$) (Fig. 5.6C). The reduced replication of the SPI-2 T3SS mutant was the main factor that accounted for the large difference in net growth between the two strains (Fig 5.6C), as no significant difference was detected between the extent of host killing undergone by wild-type and SPI-2-deficient bacteria from 0 h to 8 h p.i. (Fig 5.6D). The fluorescence dilution profiles of both strains were also similar and normally distributed (Fig. 5.6A-B), which provides further evidence that the SPI-2 T3SS-deficient strain did not incur more killing. In conclusion, these experiments confirm that the SPI-2 T3SS contributes to the replication of *S.* Typhimurium *in vivo* and that this effect begins to manifest early in infection. These results also corroborate work carried out by Shea and colleagues, in which the SPI-2 T3SS was shown to contribute preferentially to the replication of *S.* Typhimurium in the spleen, rather than resistance to host killing (Shea *et al.*, 1999).

5.2.5. Analysis of the host cell type that *S.* Typhimurium preferentially replicates within during systemic disease

Having characterised the replication of wild-type *S.* Typhimurium in the spleen, experiments were subsequently carried out to determine the host cell type that these bacteria preferentially replicate within during systemic disease. This is currently a matter of debate, as different studies have found that *S.* Typhimurium is predominantly found within macrophages (Richter-Dahlfors *et al.*, 1997, Salcedo *et al.*, 2001) or PMNs (Geddes *et al.*, 2007, Aussel *et al.*, 2011) at late stages of infection.

Mice were inoculated intravenously with approximately 800 CFU of pre-induced wild-type *S.* Typhimurium carrying pFCcGi. Animals showed signs of illness (hunched posture, ruffled fur and laboured movements) by day 5 p.i., at which time they were sacrificed and the spleens were removed. In each experiment, the spleens from 2 infected mice were pooled together. These were homogenised in the absence of detergent to avoid cell lysis and $CD11b^+$ cells were isolated using magnetic bead cell separation. In accordance with

other reports, splenic CD11b$^+$ cells were found to contain the majority of *S.* Typhimurium (Fig. 5.7A) (Matsui *et al.*, 2000). Since CD11b$^+$ is a common phagocyte marker found on both tissue macrophages and granulocytes, nuclear morphology was used to distinguish between these two host cell types. To do this, CD11b$^+$ cells were seeded onto lysine-coated coverslips, stained with a nuclear dye, DRAQ5, and examined by microscopy. The nuclei of PMNs were classified as lobed and macrophages as non-lobed (Fig. 5.7B) (Xu *et al.*, 2005, Geddes et al., 2007, Egorina *et al.*, 2008). CD11b$^+$ splenocytes were simultaneously labelled with anti-F4/80 antibodies, followed by secondary antibodies conjugated to a green fluorescent dye. F4/80 is widely used as a marker of mature macrophages (Austyn and Gordon, 1981), therefore this was used as a secondary marker to identify macrophages. Non-specific background labelling was controlled for by using infected splenic CD11b$^+$ cells labelled with a IgG2b isotype control and the same cells labelled with secondary antibody alone. In these cases, negligible or very low levels of labelling were observed (data not shown), compared to infected splenic CD11b$^+$ cells, after labelling with anti-F4/80 (Fig. 5.7D). This demonstrated that F4/80 labelling was specific.

Examination of the nuclear morphology of infected CD11b$^+$ splenocytes indicated that the majority (approximately 72%) of cells had non-lobed nuclei (Fig. 5.7B). Furthermore, approximately 62% of these infected non-lobed, CD11b$^+$ splenocytes also expressed F4/80 (Fig. 5.7C-D). Bacteria were considered intracellular if they were positioned entirely within the boundary of the host cell plasma membrane, which was visualised by using DIC imaging alongside fluorescence microscopy. However, as this technique can only visualise the cell on the x and y planes, 1 µm-thick Z stacks at 0.4 µm increments were acquired on randomly chosen cells, which confirmed that the vast majority (18 out of 20) were clearly intracellular (Fig. 5.7E). Therefore, these findings suggest that *S.* Typhimurium preferentially colonises splenic macrophages. Next, to determine which host cell type *S.* Typhimurium preferentially replicates within, the numbers of intracellular bacteria within each infected CD11b$^+$ cell were calculated. Over 60% of the total population of infected CD11b$^+$ splenocytes contained 1 or 2 bacteria per cell (Fig. 5.7F),

which is in accordance with other reports that have examined tissue sections or cell suspensions from the liver and spleen, after infection of mice with wild-type *S.* Typhimurium (Sheppard *et al*., 2003, Thone *et al*., 2007). The number of intracellular bacteria was subsequently assessed with respect to host cell type. To ensure that only PMNs and macrophages were considered, only $CD11b^+$, $F4/80^-$ cells with lobed nuclei (PMNs) and $CD11b^+$, $F4/80^+$ cells with non-lobed nuclei (macrophages) were scored. Over 80% of PMNs contained 1 or 2 bacteria per cell (Fig. 5.7F). However, enumerating the density of *S.* Typhimurium within macrophages revealed that this cell type contained many more bacteria (Fig. 5.7F). Significantly more macrophages contained 3 to 5 ($p < 0.05$), 6 to 10 ($p < 0.01$) and more than 10 ($p < 0.01$) bacteria per cell (Fig. 5.7F). In addition, PMNs were never observed to contain more than 10 bacteria per cell. This provides strong evidence that *S.* Typhimurium preferentially replicates within macrophages. In addition, all intracellular bacteria analysed had very low or undetectable levels of green fluorescence (Fig. 5.7D), indicating that these bacteria had been through 6 divisions or more. This is consistent with earlier results, which suggested that intrabacterial GFP would probably have diluted to undetectable levels shortly after 8 h p.i. (Fig. 5.6C). In conclusion, these data demonstrate that *S.* Typhimurium preferentially colonises and replicates within macrophages in the spleen during systemic infection of mice.

Experiments were also conducted as above, with *Δssa*V *S.* Typhimurium, to investigate the contribution of the SPI-2 T3SS to bacterial growth in different cell types in the spleen. In comparison to wild-type *S.* Typhimurium-infected mice, animals that were challenged with the same dose of *Δssa*V strain appeared perfectly healthy by day 5 p.i. Additionally, dissected spleens of these mice were not enlarged and no bacteria were detected by microscopy or CFU (data not shown). These findings are in agreement with previous publications showing that the SPI-2 T3SS is important for the growth of *S.* Typhimurium in the spleen of mice (Shea *et al.*, 1999). However, because no infected cells were detected under these conditions, it was not possible to analyse the numbers of intracellular *Δssa*V bacteria. Therefore, this requires

further study, possibly by using higher doses of inocula and at earlier time-points.

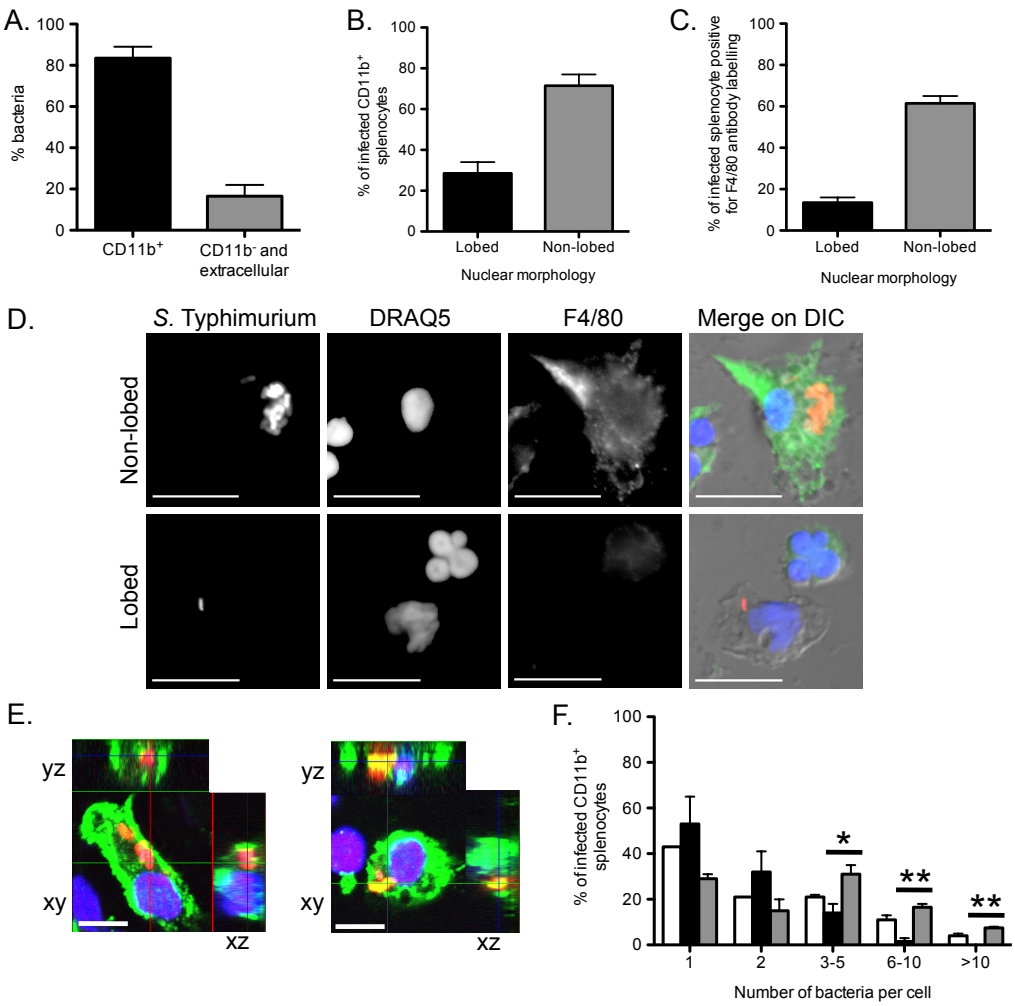


Figure 5.7. Identification of host cell types that *S.* Typhimurium colonises in the spleen. Mice were inoculated intravenously with 800 CFU of pre-induced wild-type *S.* Typhimurium carrying pFCcGi. On day 5 p.i., spleens were removed and CD11b$^+$ cells were isolated. (A) The proportion of bacteria contained within CD11b$^+$ splenocytes or CD11b$^-$ splenocytes plus extracellular bacteria. (B) Proportion of infected CD11b$^+$ splenocytes with lobed or non-lobed nuclei. (C) Proportion of infected lobed or non-lobed CD11b$^+$ splenocytes that expressed F4/80. (D) Representative microscopic images of a non-lobed and lobed CD11b$^+$ splenocytes infected with *S.* Typhimurium (red) and labelled with DRAQ5 (blue) and anti-F4/80 (green). Scale: 10 µm. (E) Representative images showing projections of xy, yz and xz planes taken from 1 µm thick Z-stacks of infected CD11b$^+$ splenocytes. Scale: 5 µm. (F) Proportion of total (white), F4/80$^-$ lobed (PMNs; black) or F4/80$^+$ non-lobed (macrophages; grey) infected CD11b$^+$ splenocytes that contain different numbers of intracellular bacteria. Groups of 2 mice were used in each experiment. (n=100 infected cells). Data represents the mean ± SEM for 2 independent experiments. Data were analysed using a non-paired, one-tailed student's t test. Values that were significantly different are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**).

5.3. Summary

To gain a better understanding of how *S.* Typhimurium colonises host tissues during disease, I applied fluorescence dilution to study the spatiotemporal replication dynamics of *S.* Typhimurium during systemic infection of mice, following the natural, oral route of inoculation. These experiments revealed, for the first time, that replication is not required for *S.* Typhimurium to traverse the intestinal wall and reach systemic tissues, including the spleen. Surprisingly, the MLN were found to be seeded initially by bacteria that have not replicated, while replication had already started in the PP. Indeed, the replication dynamics of *S.* Typhimurium appeared to be more restricted in the MLN, compared to the PP. In contrast to the MLN, the spleen was preferentially seeded by bacteria that had already undergone several divisions. Further investigation of the replication dynamics of *S.* Typhimurium in the spleen, using i.v. inoculation, confirmed that the SPI-2 T3SS contributes significantly to bacterial replication at this site and that within this organ, wild-type *S.* Typhimurium preferentially colonises and replicates within macrophages.

6. DISCUSSION

An area of increasing interest is the study of growth and dissemination of *S.* Typhimurium *in vivo*. This is fundamental to understanding disease pathogenesis, particularly with respect to the colonisation of host tissues. During this study, fluorescence dilution was developed as a tool to measure bacterial replication directly at the population and single cell levels. This technique was subsequently used to study the spatiotemporal replication dynamics of *S.* Typhimurium during systemic disease in mice.

6.1. Fluorescence dilution as a tool to measure bacterial replication

As a result of experiments carried out in Chapter 3, fluorescence dilution was established as a robust and versatile technique. I used several fluorescent proteins (DsRed, GFP and mCherry) as reporters of bacterial replication and showed that measurements of replication, obtained by fluorescence dilution with any of these fluorescent proteins, were comparable to CFU enumeration. Furthermore, the simultaneous dilution of two fluorescent proteins *in vitro* was also equivalent. The dynamic range of measurement using a single fluorescent protein was limited to 6 generations of replication. However, this could be increased to 10 generations, by sequentially diluting a second fluorescent protein. Sequential fluorescence dilution can easily be applied to tissue culture models and a dynamic range of up to 10 generations (over 1000 fold) is more than enough to adequately monitor *S.* Typhimurium replication in these systems. However, this might not be as easily applied to *in vivo* models (discussed below).

Fluorescence dilution was first developed *in vitro*, using pDiGc. However, this plasmid was found to impart a significant invasion and replication defect on *S.* Typhimurium, which prevented the use of this plasmid for *in vivo* studies, primarily because invasion of the intestinal epithelium is required for *S. enterica* to efficiently establish a systemic infection in mice (Galan *et al.*, 1989). Indeed, it has been shown that plasmid carriage and fluorescent protein expression can affect bacterial activities, including invasion efficiency

and survival inside host cells (Wendland *et al.*, 2002, Rang *et al.*, 2003, Coulson *et al.*, 1994, Abromaitis *et al.*, 2005, Knodler *et al.*, 2005, Clark *et al.*, 2009). Therefore, a major element of the work described in Chapter 3 was the development of pFCcGi, an optimised reporter system, to overcome these limitations. *S.* Typhimurium carrying pFCcGi invaded non-phagocytic cells efficiently *in vitro* and, despite being recovered from the spleen in moderately lower numbers than the wild-type strain on day 3 post-oral inoculation of mice, this strain efficiently colonised spleens by day 6. The decreased fittness cost of pFCcGi over pDiGc is most likely due to its lower copy number, based on the copy number of pFPV25 (10-30 copies per cell) (Valdivia and Falkow 1996) compared to pBAD18 (50-100 copies per cell) (Guzman *et al.*, 1995). In addition, pFCcGi lacks DsRed, which was suggested to attenuate the virulence of *S.* Typhimurium *in vivo* (Prof. D. Bumann, personal communication). This is potentially due to the tetrameric nature of DsRed, which could lead to aggregation within bacterial cells and disruption of cellular functions. When used in mixed infection experiments with the wild-type strain, a mild virulence attenuation of *S.* Typhimurium carrying pFCcGi was apparent. However, this level of attenuation was comparable to strains carrying the widely used plasmid, pFPV25.1 (Knodler *et al.*, 2005a). Importantly, in experiments where the replication rates of wild-type and mutant strains are compared, such as those carried out in Chapter 5 with SPI-2 T3SS-deficient bacteria, all strains carry the fluorescence-encoding plasmid. Therefore, differences due to the mutation(s) in question should still be apparent.

Applying fluorescence dilution to bacteria in an *in vivo* model raised the question of how stresses present within this system might affect intrabacterial fluorescent protein stability. In particular, it was crucial to assess whether there was replication-independent loss of GFP fluorescence, which could lead to incorrect interpretation of green fluorescence loss. Within the host, bacteria are exposed to gastric acid in the stomach and low oxygen tension within the intestinal lumen: conditions well known to affect fluorophore formation (Heim *et al.*, 1994, Marteyn *et al.*, 2010, Doherty *et al.*, 2010). Other authors have described deficient or partial fluorescence of GFP, when synthesised by

bacteria in the intestine, but not in systemic organs (Rollenhagen *et al.*, 2006, Marteyn *et al.*, 2010). For example, Rollenhagen and colleagues demonstrated that the fluorescence of GFP synthesised by *S.* Typhimurium in the caeca of mice could be enhanced by approximately 33% after a further incubation step in aerated medium in the presence of chloramphenicol to block *de novo* synthesis of GFP. However, no further increase was observed when bacteria were recovered from the spleen. In contrast to these investigations, I was concerned with the stability of *pre-formed* intrabacterial GFP *in vivo*. However, my findings are consistent with these reports. Experiments described in Chapter 4, using pFCiGi, revealed that 40% and 30% replication-independent loss of intrabacterial GFP fluorescence did occur *in vivo*, in bacteria recovered from the ileal lumen and PP, respectively (Table 4.1). However, no evidence of GFP instability was observed in bacteria recovered from the MLN and spleen, which also support the conclusion that the replication-independent loss of green fluorescence observed in *S.* Typhimurium recovered from the SI and PP is due to exposure to some environmental stress that bacteria are exposed to in the gut. These results conflict with a previous suggestion that the fluorescence of GFP synthesised by *S.* Typhimurium prior to entering the host is not altered when recovered from the caecum (Rollenhagen *et al.*, 2006). The reason for this is unclear. In experiments described in Chapter 5, I excluded replication-independent loss of GFP fluorescence from analysis: replication-independent loss of GFP fluorescence produced heterogeneous profiles, each with a low level of green fluorescence intensity, whereas bacterial replication produced homogeneous profiles, each with a normal-distribution of green fluorescence intensity (Fig. 5.2).

The identification of replication-independent loss of GFP fluorescence in bacteria recovered from the intestinal lumen and PP indicates that a proportion of bacteria in these sites has been subjected to environmental stresses. Therefore, pFCiGi provides a GFP sensor to detect bacterial stress in *S.* Typhimurium *in vivo*. Fluorescence biosensors developed to detect specific stresses to which *S.* Typhimurium is exposed, include that of the OxyR-dependent *ahpC-gfp* fusion, which senses oxidative stress (Aussel *et*

al., 2011). Aussel and colleagues used this system to show that *S.* Typhimurium is exposed to oxidative stress within vacuoles in macrophages.

Exposure to low ambient pH and subsequent loss of intracellular pH homeostasis, rather than oxygen-limitation, most likely resulted in the replication-independent loss of GFP fluorescence *in vivo*, as *in vitro* experiments indicated that intrabacterial GFP was stable for up to 24 h after complete removal of oxygen (Fig. 4.3C). In contrast, GFP lost fluorescence within 5 min, when *S.* Typhimurium was exposed to an ambient pH of 2.0 (Fig. 4.3A). *S.* Typhimurium does possess acid tolerance response (ATR) systems to protect itself against acid stress and maintain a relatively neutral internal pH. ATR systems are controlled by regulatory genes, including the alternative sigma factor encoded by *rpoS*, the iron regulator *fur*, the *phoPQ* regulon and the *ompR* response regulator (Foster, 1995, Foster, 1999). These are typically activated during growth in a moderately acidic environment and induce the synthesis of acid shock proteins, which protect the cell against more extreme acid conditions. The ATR enables cells to withstand a pH of approximately 3.3 (Foster and Hall, 1990). However at increased acidic conditions, loss of intracellular pH homeostasis rapidly leads to bacterial cell death (Peterson *et al.*, 1989). Since hydrochloric acid in the stomach can generate conditions as acidic as pH 1 to 2 (Peterson *et al.*, 1989), bacteria were administered to mice in an alkaline buffer. Nevertheless, the results described in Chapter 4 suggest that this buffer was insufficient to neutralise stomach acidity.

Following the validation, optimisation and adaptation of fluorescence dilution to measure bacterial replication *in vitro* and *in vivo*, this technique was used to study *S.* Typhimurium replication both in macrophages and in mice. This revealed several important insights into the pathogenesis of *S. enterica* that I will discuss below. However, this work also identified a number of limitations of the technique, namely the restricted dynamic range of measurement *in vivo* to 6 generations of replication and the replication-independent loss of GFP fluorescence within the gastrointestinal tract. The former limitation did not present a significant problem in tissue culture models, as sequential fluorescence dilution can be used to increase the dynamic range of

measurement to over 10 generations, furthermore the inducer can easily be maintained and removed when desired. However, this might not be as straight-forward in mice, since an externally administered inducer would have to penetrate deep into tissues to reach bacteria and in addition, fluorescent protein synthesis would have to be terminated by all bacteria in a reasonably synchronous manner. The issue of GFP instability *in vivo* could potentially be overcome by using mCherry, which I showed to be more stable, instead of GFP. However, this would require the generation of a mCherry variant, for example by using site-directed mutagenesis, that provides a greater fluorescence yield.

6.2. Intracellular replication of *S.* Typhimurium within macrophages *in vitro*

Fluorescence dilution was used initially to characterise the replication dynamics of *S.* Typhimurium within a RAW264.7 macrophages. This demonstrated that bacteria can replicate approximately 400 fold (8 generations) over 16 h inside these cells. From 2 h to 6 h post-uptake, bacteria replicate slowly, which could be due to nutrient deprivation (Appelberg, 2006) and exposure to oxidative stress (Vazquez-Torres *et al.*, 2000a). Replication increases to a rate of 1 division per h, from 6 h onwards. This corresponds to the time at which SPI-2 T3SS-associated genes are maximally expressed (Cirillo *et al.,* 1998) and when certain SPI-2 T3SS-translocated effectors can be detected within host cells (Kuhle and Hensel, 2002). These effectors subsequently create a more favourable environment for replication, by modifying the SCV (Waterman and Holden, 2003). Indeed, the replication defect of SPI-2 T3SS mutants becomes apparent 6 h after uptake by RAW264.7 macrophages (Helaine *et al.*, 2010), suggesting that the increased replication rate from 6 h onwards is due to the activities of the SPI-2 T3SS effectors. Interestingly, *S.* Typhimurium was found to replicate at approximately the same rate during exponential growth in MM and within RAW264.7 macrophages. This indicates that bacteria are very well adapted to life within this host cell type. From 16 h onwards, it is possible that bacteria continue to proliferate at high rates until the host cell ruptures. Alternatively,

replication may begin to subside as the host cell becomes limiting for nutrients. Indeed, mathematical modelling has indicated that the intracellular replication of *S.* Typhimurium decreases as the intracellular bacterial load increases (Brown *et al.*, 2006). Since fluorescence dilution provides analysis of single host cells, this technique could provide a means to test this hypothesis of intracellular growth restriction.

6.3. Spatiotemporal replication dynamics of *S.* Typhimurium during systemic disease in mice

6.3.1. Replication and systemic dissemination

S. Typhimurium uses several routes to traverse the intestinal barrier, mainly at the level of the distal ileum, and gain access to systemic sites (Fig. 1.5). SPI-1-mediated invasion of enterocytes and particularly, M cells that overlie the PP, represent the predominant route of intestinal traversal (Galan *et al.*, 1989, Jones *et al.*, 1994, Clark *et al.*, 1994). Following this, a subpopulation of *S.* Typhimurium from the PP and/or lamina propria is presumably transported to and colonises the draining MLN. Experiments described in Chapter 4 suggest that initial colonisation of the MLN is an active process, as only unstressed bacteria were found in these tissues soon after oral inoculation of mice (Table 4.1). It is possible that stressed bacteria are less able to activate a virulence mechanism, such as SPI-1 T3SS-mediated invasion of the intestinal wall, which facilitates seeding of the MLN. On the other hand, this finding may simply indicate that stressed bacteria are unable to reach the MLN, as they are killed in the intestine and/or PP.

Large numbers of bacteria can only be detected in the liver and spleen after an initial increase in bacterial numbers in the PP and MLN (Carter and Collins, 1974). Therefore, it seems likely that a subpopulation of replicating bacteria from the PP and/or MLN disseminates further via the lymphatics and bloodstream, to eventually seed the liver and spleen. In support of this, the majority of bacteria found to populate the spleen following oral inoculation had already undergone several divisions and at these times, bacterial replication

was observed within the PP and MLN (Fig. 5.2). However, extensive intracellular replication of bacteria does not appear to be essential for systemic dissemination, as mutants that are unable to replicate inside host cells can still disseminate from the gut to the liver and spleen (Leung and Finlay, 1991, Cheminay et al., 2004). Indeed, findings presented in Chapter 5 show that bacteria that have not replicated are able to traverse the intestinal epithelium and seed the PP, MLN and spleen (Fig. 5.2). Interestingly, this suggests that *S.* Typhimurium is capable of uncoupling virulence gene expression from bacterial replication *in vivo*. Growth-phase has been suggested to regulate *S.* Typhimurium virulence traits. Some genes, for example those associated with the SPI-2 T3SS, are upregulated during stationary-phase growth (Guiney *et al.*, 1995, Beuzon et al., 1999). Therefore, certain virulence genes are preferentially expressed during conditions of low bacterial growth. However to my knowledge, the expression of *S.* Typhimurium virulence genes in the absence of bacterial replication, has not yet been described.

Despite having a reduced ability to traverse the intestinal epithelium, SPI-1 mutants are still eventually able to reach the MLN and systemic organs following oral inoculation (Galan and Curtiss, 1989, Baumler *et al.*, 1997b, Murray and Lee, 2000), suggesting that invasion-independent routes of dissemination exist. Consistent with this, studies have identified routes of dissemination that do not require prior colonisation of PP. Vazquez-Torres and colleagues demonstrated that a SPI-1 mutant strain of *S.* Typhimurium was able to bypass the PP and seed the spleen directly from the intestinal lumen, via the bloodstream (Vazquez-Torres *et al.*, 1999). A later study showed indirectly that invasive *S.* Typhimurium also disseminates via this route (Worley *et al.*, 2006). The results in Chapter 5 illustrated the possibility of a direct route of bacterial dissemination from the gut to the spleen: non-replicating bacteria were found to reach the spleen following oral inoculation of mice and non-replicating bacteria were found to persist predominantly within the ileal lumen (Fig. 5.2 and Table 5.1). However, non-replicating bacteria were also detected in the PP and MLN, therefore it is equally as likely that non-replicating bacteria detected in the spleen were derived from the PP

and MLN. Nevertheless, it would be interesting to investigate if the same processes mediate the migration of replicating and non-replicating bacteria towards the spleen. Indeed, SPI-1-dependent and independent seeding of the spleen involves distinct processes, which appear to differ in efficiency and timing.

The contribution of different routes of bacterial dissemination to eventual colonisation of the spleen by *S.* Typhimurium is not fully understood. Interestingly, a PP-independent route is evident in yersiniosis and this represents the main route of dissemination of *Yersinia* to the spleen and liver (Barnes *et al.*, 2006). Barnes and colleagues elegantly demonstrated that a lethal infection of mice was dependent on a wave of *Yersinia* that disseminate to the spleen via the bloodstream, from a replicating pool of bacteria in the gut. It was suggested that these bacteria might be more appropriately primed to colonise the spleen and liver following prior and prolonged replication in the intestine (Barnes *et al.*, 2006). In contrast to this, my results suggest that an alternative pathway of *S.* Typhimurium dissemination to the spleen might stem from a non-replicating population of bacteria.

6.3.2. Rapid bacterial replication within the PP

Fluorescence dilution revealed that *S.* Typhimurium began to replicate in the PP within 2 h of infection (Fig. 5.2). In addition, this replication was relatively rapid (up to 1 division every h). Replication of *S.* Typhimurium was not observed within the intestinal lumen at this time, suggesting that bacteria only began to replicate upon invasion of the PP, presumably once they had reached a permissive intracellular compartment. However, microscopy would be required to establish unequivocally whether this replicating population is intracellular. If so, it is interesting to consider which host cell type this early replication might be taking place within. M cells often contain several intracellular bacteria (Jones *et al.*, 1994), therefore it is likely that *S.* Typhimurium replicated rapidly upon invasion of these host cells. Interestingly, despite the identification of replicating bacteria in the PP at 2 h and 6 h p.i., in most cases at these time-points, the majority of bacteria had not replicated yet

(Table 5.1). Bacterial replication in the PP could be host cell type dependent. It has been reported that subepithelial DCs are the first cell type to take up *S.* Typhimurium in the PP (Hopkins *et al.*, 2000) and in addition, *S.* Typhimurium is not thought to replicate efficiently within cultured DCs *in vitro* (Niedergang *et al.*, 2000, Jantsch *et al.*, 2003, Bueno *et al.*, 2008). Therefore, it is possible that DCs preferentially contain non-replicating bacteria in the PP and replicating bacteria reside within other cell types. Indeed, another study reported that PP $CD11c^+$ cells typically contain single bacteria after oral inoculation of mice, whereas clusters of replicating bacteria were observed within $CD11c^-$ cells (Cheminay *et al.*, 2005). However, at 12 h p.i., I found bacteria that had replicated within $CD11c^+$ cells in the PP, as well as the $CD11b^+$ and unlabelled cell fractions, suggesting that replication might also have occurred within DCs. Microscopy could be used to distinguish if bacteria had replicated within DCs or if these cells phagocytosed replicating bacteria: one replicating bacterium per cell would suggest that replication occurred elsewhere.

6.3.3. Restricted bacterial replication within the MLN

Fluorescence dilution revealed that the dynamics of *S.* Typhimurium replication in different murine tissues were not equivalent. Experiments described in Chapter 5 indicated that bacterial replication was more restricted in the MLN compared to the PP. This is consistent with evidence showing that the growth of *S.* Typhimurium in the MLN is not as marked as in the liver and spleen (Voedisch *et al.,* 2009). Furthermore, the MLN seem to represent a restrictive site for dissemination of *S.* Typhimurium, as mesenteric lymphadenectomised mice experienced accelerated spleen and liver colonisation and increased mortality (Voedisch *et al.,* 2009). Using the same technique, a recent study showed that the MLN also restrict the dissemination of *S.* Typhimurium during a relapsing infection (Griffin *et al*., 2011).

The MLN are important for the generation of systemic immune responses (Yamamoto *et al.*, 2000) and these tissues produce significant quantities of protective cytokines during infection with *S.* Typhimurium (Karem *et al.*, 1996).

Therefore, it is possible that mice lacking MLN develop an increased susceptibility to *S.* Typhimurium, because of a general impairment in the development of an appropriate immune response. However, lymphadenectomised $Rag2^{-/-}$ mice that lack mature T and B-cells, do not develop enhanced bacterial loads in the spleen and liver infection with *S.* Typhimurium (Voedisch *et al*., 2009). On the other hand, restricted replication and dissemination of *S.* Typhimurium within the MLN could result from an intrinsic property of these lymphoid tissues, for example due to the host cell type that bacteria are contained within. Indeed, DCs appear to represent the majority of infected cells at this site (Macpherson *et al.*, 2004, Voedisch *et al.*, 2009) and *S.* Typhimurium seems to be unable to replicate (Jantsch *et al.*, 2003, Niedergang *et al.*, 2000) or to replicate poorly (Bueno *et al.*, 2008) within this cell type. Furthermore, upon entering the lymph nodes, DCs harbouring commensal bacteria do not leave this tissue (Macpherson *et al.*, 2004). The restrictive capacity of the MLN could therefore be due to DCs that harbour the majority of bacteria. I did attempt to determine whether replication within the MLN was host cell type dependent, however unfortunately the results were inconclusive, as a large amount of error was observed between experiments.

The restriction of *S.* Typhimurium replication in the MLN and their limited dissemination from this site could represent a selective pressure favouring the exploitation of alternative pathways for bacteria to bypass the MLN and directly target more permissive environments, such as within macrophages in the liver (Richter-Dahlfors *et al.*, 1997) and spleen (Salcedo *et al.*, 2001). Bypassing certain tissues, such as the PP or MLN, might also diminish the priming of adaptive immune responses (Vazquez-Torres et al., 1999, Martinoli *et al.*, 2007). On the other hand, the reduced replication of *S.* Typhimurium in the MLN may be bacterially directed. Reducing replication may seem counterintuitive; however, several *S.* Typhimurium virulence factors have been implicated in the downregulation of bacterial growth, including: the PhoPQ two-component regulatory system (Cano *et al.*, 2001), genes involved in nitric oxide production (Eriksson *et al.*, 2000) and the D-Ala-D-Ala dipeptidase*, pcgL* (Hilbert *et al.*, 1999), which could stimulate immune

signalling (Mouslim *et al.*, 2002). Such 'antivirulence' genes are also found in other organisms and have been hypothesised to promote host survival (Cunningham *et al.*, 2001) or alternatively, to facilitate survival in secondary environments during host to host transmission (Giraud *et al.*, 2001). Reduced replication of *S.* Typhimurium within DCs could help to limit antigen presentation (Albaghdadi *et al.*, 2009) and therefore, evade immune recognition and avoid bacterial killing. Interestingly, Monack and colleagues demonstrated that *S.* Typhimurium is capable of persisting in the MLN of mice for up to one year (Monack et al., 2004a). Therefore, it is possible that bacteria could also exploit the restrictive capacities of these tissues or specifically downregulate replication within this site to form reservoirs of persistent bacteria. In light of my findings, it would be interesting to investigate whether the restrictive nature of *S.* Typhimurium replication within the MLN is even more pronounced in the murine model of chronic typhoid fever.

3.4. Bacterial growth dynamics in the spleen

Bacterial growth and organ colonisation during murine typhoid is mostly studied within the spleen and liver, as these represent the major sites of systemic bacterial replication. Inoculation of mice with *S.* Typhimurium via the oral route provided insights into how bacterial replication relates to initial seeding of the spleen. However, to investigate the detailed dynamics of population growth at this site, the i.v. route of inoculation had to be used, to facilitate direct seeding of the spleen and to circumvent the problem of the limited range of cell divisions that could be measured by fluorescence dilution within this organ after oral inoculation.

Following i.v. inoculation, the replication of *S.* Typhimurium in the spleen proceeded in a relatively homogeneous manner. This contrasted with the typically heterogeneous profile of bacterial replication observed within murine tissues following oral inoculation. The constant entry and exit of bacteria from an individual tissue creates greater heterogeneity within an individual population of bacteria (Grant *et al.*, 2008) and this probably explains the heterogeneous profiles observed after oral inoculation. In the same way, at

the early time-points investigated after i.v. inoculation, spread of bacteria between organs is unlikely (Grant *et al.*, 2008).

The precise contribution of bacterial replication and killing to overall net growth of *S.* Typhimurium during initial colonisation of the spleen was determined by coupling measurements of bacterial replication obtained from fluorescence dilution and net growth from CFU; these were then used to generate a killing index (Helaine *et al.*, 2010). These experiments demonstrated that high rates of bacterial replication took place soon after reaching the spleen, despite the onset of rapid bacterial killing. The dynamics of replication and killing, in addition to the actual rates obtained with fluorescence dilution, are consistent with results obtained using DNA-tagged, isogenic strains of *S.* Typhimurium (WITS) (Grant *et al.*, 2008). These authors also used Phox-deficient mice to demonstrate that this early killing is mediated by the oxidative burst. Following this, a second phase occurred, characterised by negligible killing and moderate division rates, which is also in accordance with experiments using WITS (Grant *et al.*, 2008). In the WITS model, this temporal change in growth dynamics was attributed to a shift in the role of oxidative stress from bactericidal to bacteriostatic, as the mean generation time of *S.* Typhimurium in Phox-deficient mice was more than half that observed in wild-type mice, from 6 h onwards when killing was negligible. Transfer of *S.* Typhimurium from PMNs to macrophages, a more permissive cell type, could potentially account for this observation. However, an increased replication rate of *S.* Typhimurium was not observed by fluorescence dilution in Phox-deficient bone marrow-derived macrophages, compared to their wild-type counterparts (Dr. S. Helaine, personal communication).

The replication of *S.* Typhimurium in the spleen was also measured by using a temperature-sensitive plasmid, in previous work by our group (Shea *et al.*, 1999). In this report, the i.p. route of inoculation was used. After 16 h of infection, wild-type *S.* Typhimurium was found to have undergone 4 (intracellular) or 5 to 6 (extracellular) generations of replication. Intra- and extracellular populations of bacteria were not distinguished in my

experiments. However, since the replication profile of the pooled populations of bacteria were relatively homogeneous (Fig. 5.6A-B), this does not provide evidence of two distinct populations of replicating bacteria. Nonetheless, it is possible that a difference in replication between intra- and extracellular bacteria becomes more pronounced as the infection progresses. In the WITS model, bacteria were found to divide every 1.7 h between 0.5 h to 6 h p.i. and every 8 h between 6 h to 24 h p.i. (Grant *et al.*, 2008). Therefore, this suggests that at 16 h p.i. bacteria would have undergone approximately 4.5 divisions. *S.* Typhimurium replication in the spleen after i.v. inoculation has also been measured by Maw and Meynell using the dilution of a superinfecting phage (Maw *et al.*, 1968). Bacteria were found to replicate through 1 generation by 1.5 h and a further 10 generations by 48 h p.i., therefore this suggests that bacteria replicate through approximately 4 generations over 16 h of infection. Therefore, three independent methods (plasmid dilution, WITS and phage dilution) to measure *S.* Typhimurium replication, using different routes of inoculation (i.p. and i.v.), yield similar results. These findings demonstrate that *S.* Typhimurium replicates within the spleen at a similar rate, despite having reached this organ via different routes.

3.5. The contribution of the SPI-2 T3SS to bacterial replication in the spleen

SPI-2 T3SS-deficient strains of *S.* Typhimurium are competent for invasion of non-phagocytic cells (Ochman *et al.*, 1996) and after oral inoculation, these bacteria are able to reach the PP (Cirillo *et al.*, 1998, Cheminay *et al.*, 2004). However, without a functional SPI-2 T3SS, *S.* Typhimurium cannot efficiently colonise the PP, MLN, spleen and liver of mice to develop a systemic disease (Shea *et al.*, 1996, Cirillo *et al.*, 1998, Shea *et al.*, 1999, Cheminay *et al.*, 2004). Previous work from our group demonstrated that from 4 h to 8 h post-i.p. inoculation, *Δssa*V and wild-type bacteria undergo a small increase in net growth in the liver and spleen (Shea *et al.*, 1999). However, as the net growth of wild-type bacteria continued to increase, the numbers of the mutant remained constant over the next week, then began to gradually decrease. After 4 months, most mice had cleared the SPI-2 T3SS-deficient strain (Shea

et al., 1999). Consistent with these results, there was a slight increase in net growth of wild-type *S.* Typhimurium from 2 h to 8 h post-i.v. inoculation in my experiments. However, there was a decrease in net growth of the SPI-2 T3SS-deficient strain over the same period of time (Fig. 5.6C), which contrasts with earlier findings. Differences in the experimental conditions used in the two studies could explain the discrepancies in cell counts, including the dose and route of inoculation. In my experiments, mice were inoculated intravenously with 800 CFU, whereas Shea *et al.* inoculated mice intraperitonally with 10^5 CFU of bacteria.

Reduced net growth *in vivo* could be due to a defect in replication or an increased susceptibility to host killing, or both. However, by using the temperature-sensitive plasmid to calculate the rate of bacterial replication and killing in the spleen, the reduced growth of SPI-2 T3SS mutants was found to be predominantly due to a defect in replication, rather than resistance to host killing (Shea *et al.*, 1999). An inability to replicate within macrophages was also suggested by other work from our group, which demonstrated that after 3 days of infection, less than 5% of SPI-2 T3SS mutant-infected splenocytes contained more than 5 bacteria per cell, whereas approximately 45% of wild-type-infected splenocytes contained more than 5 bacteria per cell (Salcedo *et al.*, 2001). The findings by Salcedo *et al.* also suggested that the SPI-2 T3SS-deficient bacteria were not more susceptible to host killing, as the number of splenocytes infected with this strain remained constant over 5 days of infection. My results confirm these observations, showing that SPI-2 T3SS mutants replicate in the spleen to a significantly lesser extent than wild-type bacteria and this was not accompanied by a significant difference in host killing sustained by both strains. Results consistent with this were also obtained by Dr. S Helaine of our group, who used fluorescence dilution and CFU to examine *S.* Typhimurium replication and killing within bone marrow-derived macrophages (Helaine *et al.*, 2010).

Other reports have implicated the SPI-2 T3SS in the avoidance of SCV-lysosome fusion (Uchiya *et al.*, 1999) and Phox- (Vazquez-Torres *et al.*, 2000b), and iNOS- (Chakravortty *et al.*, 2002) mediated killing. My results and

other work from our group (Shea *et al.*, 1999, Salcedo *et al.*, 2001, Helaine *et al.*, 2010) contradict these findings, in particular the role of SPI-2 in resistance to the respiratory burst, as this accounts for the early host killing experienced by *S.* Typhimurium in the spleen (Grant *et al.*, 2008). In addition, a recent study reported that, although the spleens of *phox*-knockout mice contained higher loads of a SPI-2 T3SS mutant than those from wild-type mice, CI tests indicated that the mutant was similarly attenuated in both mouse strains, compared to wild-type *S.* Typhimurium (Aussel *et al.*, 2011). In conclusion, my results add to an increasing body of evidence that indicates that the SPI-2 T3SS contributes to replication of *S.* Typhimurium *in vivo* and not resistance to host killing.

Although the SPI-2 T3SS is required for replication within and eventual colonisation of host tissues, it is clear that a SPI-2 T3SS-deficient mutant is still capable of limited growth *in vivo* (Shea *et al.*, 1999, Salcedo *et al.*, 2001). This seems most prominent during the early stages of infection in the spleen (Fig. 5.6C), which is surprising given that the SPI-2 T3SS is maximally expressed 6 h following uptake by cultured cells (Cirillo *et al.*, 1998). However, the temporal activation of the SPI-2 T3SS is not known *in vivo*. In addition, *S.* Typhimurium has been shown to replicate independently of the SPI-2 T3SS within some cultured cell-lines for at least the first 6 h post-uptake (Brumell *et al.*, 2001, Helaine *et al.*, 2010).

For the experiments discussed in this section, further insights could clearly be obtained by extending the dynamic range of measurement of fluorescence dilution. This is because SPI-2 T3SS-translocated effectors are only detected within host cells after several hours of infection and SPI-2-dependent replication only becomes apparent 6 h following uptake by cultured macrophages. Therefore, the functions of the SPI-2 T3SS are likely to be more pronounced later in infection. Fluorescence dilution could be used in future work to investigate the role of other bacterial factors, such as the SPI-1 T3SS and the PhoPQ regulon, and host factors, such as the Slc11a1, Phox and iNOS, on *S.* Typhimurium growth during systemic disease. How bacterial replication (or lack of replication) relates to the expression of specific genes

could also potentially be investigated by combining fluorescence dilution with additional fluorescent protein reporters that are encoded downstream of target gene promoters. It would also be interesting use fluorescence dilution to determine how bacterial growth relates to host-specific disease progression by different *S. enterica* serovars (Paulin *et al.*, 2007).

6.4. *S.* Typhimurium preferentially colonises and replicates within splenic macrophages

Despite extensive study of the growth dynamics of *S.* Typhimurium in the liver and spleen, the intracellular localisation of replicating bacteria within these organs is still debated. It is generally accepted that *S.* Typhimurium preferentially colonises macrophages *in vivo*. This is partly because the ability of *S.* Typhimurium to survive and replicate intracellularly within macrophages correlates with virulence in mice (Fields *et al.*, 1986) and macrophage-depleted mice display reduced bacterial loads (Wijburg *et al.*, 2000). In addition, several studies, using microscopic or flow cytometric analysis, have shown that *S.* Typhimurium is contained predominantly within cells labelled with macrophage markers in the liver (Richter-Dahlfors *et al.*, 1997) and spleen (Salcedo *et al.*, 2001), during late stages of systemic disease. Further characterisation of infected splenocytes, by our group, revealed that *S.* Typhimurium localises to specific subsets of marginal zone and red pulp macrophages (Salcedo *et al.*, 2001). However, these conclusions have been challenged recently by two other groups, which instead found that the majority of *S.* Typhimurium is contained within splenocytes labelled with antibodies specific for PMNs, at late stages of infection (Geddes *et al.*, 2007, Aussel *et al.*, 2011). Furthermore, one such study also used morphologic analysis on a portion of these antibody-labelled cells, after fluorescence-activated cell sorting (FACS), to confirm their identity (Geddes *et al.*, 2007). This is surprising, since PMNs are thought to be relatively short-lived and appear to kill *S.* Typhimurium efficiently (Conlan, 1997).

In experiments described in Chapter 5, *S.* Typhimurium was found within macrophages and PMNs in the spleen, at late stages of infection. However,

the majority of infected cells resembled macrophages (distinguished as non-lobed, F4/80$^+$, CD11b$^+$ splenocytes), suggesting that *S.* Typhimurium preferentially colonises this host cell type *in vivo*. It is possible that a proportion of the infected macrophages identified in my study might represent DCs, as F4/80 was found to be expressed by these cells (Leenen *et al.*, 1998). Indeed, other studies have shown that *S.* Typhimurium infects splenic DCs in mice (Yrlid *et al.*, 2001, Yrlid and Wick, 2002). However, the experimental conditions used in these studies differed greatly from my work. Yrlid *et al*., inoculated mice intraperitoneally or intravenously with very large numbers of bacteria (10^8 to 10^9 CFU) and recovered infected cells after 4 h, whereas I inoculated mice intravenously with 800 CFU and sacrificed mice after 5 days. To exclude the possibility of including DCs as macrophages in future experiments, additional macrophage-specific markers, such as scavenger receptors or MOMA-2 could be used (Leenen *et al.*, 1994, Peiser *et al.*, 2002). A potential explanation for the contradictory results of my study, and work by Richter-Dahlfors *et al.* and Salcedo *et al*., to the results of Geddes *et al*. and Aussel *et al*., who found that the majority of infected cells represented PMNs, could be the time of infection in which the respective investigations were carried out. Although in all studies, mice were sacrificed when the disease had reached an advanced stage, Geddes *et al*. and Aussel *et al*. inoculated mice with higher doses of bacteria (>10^5 CFU) and analysed infected splenocytes after a shorter period of infection (2 days). Therefore, the host cell type that *S.* Typhimurium colonises *in vivo* may be very dependent on the inoculum dose, stage of disease and time of infection. For example, it is possible that *S.* Typhimurium replicates within different host cell types as they become available and PMN influx to infection foci preceeds the influx of mononuclear cells (Nakoneczna and Hsu, 1980). Indeed, it has been previously reported that the majority of infected splenocytes after 4 h and 24 h post-i.v. inoculation represented PMNs (Dunlap *et al.*, 1992). However, it should be noted that Dunlap *et al*., used very high doses (10^6 CFU) of bacteria to inoculate mice and very few infected cells were recovered. Geddes *et al.,* also suggested that immune cell-surface expressed proteins might change during an infection, which could give rise to misleading results (Geddes *et al.*, 2007). They detected F4/80 (Austyn *et al.*, 1981) and CD115

or macrophage colony-stimulating factor receptor (Sasmono *et al.*, 2003) on both splenic monocytes and PMNs, despite the widespread assumption that these are monocyte/macrophage-specific. Therefore, in the future, it may prove valuable to clarify the expression pattern of cell-specific markers under the appropriate context in which they are to be used.

My results also demonstrated that macrophages contained significantly higher numbers of intracellular bacteria, compared to PMNs, providing evidence that *S.* Typhimurium preferentially replicates within macrophages *in vivo*. In addition, all intracellular bacteria had diluted their green fluorescence to undetectable levels, indicating that they had divided over 6 times. Examination of intracellular bacterial loads has shown that the numerical distribution of intracellular bacteria is dependent on the intrinsic bacterial growth rate and immune control mechanisms, such as the oxidative burst and IFN-γ (Grant *et al.*, 2009). Therefore, lower numbers of intracellular bacteria might be observed within PMNs, despite the occurrence of bacterial replication, as this cell type is known to be more bacteriocidal than macrophages. However, since there were 2.5 times more infected macrophages (or non-lobed cells) than PMNs (or lobed cells) (Fig. 5.7B), it is more likely that these bacteria replicated elsewhere and upon entering PMNs, replication drastically slowed or ceased altogether.

Although macrophages were found to contain higher intracellular bacterial loads compared to PMNs, the majority (64%) of all infected $CD11b^+$ splenocytes contained an average of one or two bacteria per cell and approximately 85% contained less than 5 bacteria per cell (Fig. 5.7F). These findings are consistent with other studies that have used microscopy or flow cytometric analysis to examine the intracellular loads of *S.* Typhimurium-infected cells in the liver and spleens of mice at late stages of infection (Sheppard *et al.*, 2003, Thöne *et al.*, 2007, Grant *et al.*, 2008). In contrast, our group previously found much higher numbers of intracellular *S.* Typhimurium in infected splenocytes, with approximately 45% of infected cells containing more than 5 bacteria (Salcedo *et al.*, 2001). These contradictory findings appear to be independent of the challenge dose, as low numbers of

intracellular bacteria within infected cells have been reported after inoculating mice with a wide range of CFU, from 800 CFU (this study) to greater than 10^7 CFU (Sheppard *et al*., 2003). Importantly, the main difference between the experimental conditions used by Salcedo *et al.,* compared to those used in my work and other studies, is the route of inoculation. Salcedo *et al*., inoculated mice intraperitonally whereas in my experiments and those of others, mice were inoculated by the i.v. route. Therefore, it is possible the progression of infection might differ depending on the route of inoculation. With this in mind, it might be useful to examine the intracellular loads of infected cells after oral inoculation of mice, which represents the natural route of *S. enterica* transmission.

Interestingly, although most infected cells typically harbour only one or two bacteria, several studies have found that the vast majority of *S.* Typhimurium in the liver and spleen reside within a few highly infected host cells (Salcedo *et al.,* 2001, Sheppard *et al.,* 2003, Thöne *et al*., 2007). Some infected cells have been reported to contain at least 50 bacteria (Salcedo *et al*., 2001). Experiments described in Chapter 5 indicated that high loads of intracellular bacteria were exclusively within macrophages and splenocytes containing more than 10 bacteria represented only approximately 4% of all infected cells. How these two populations of abundant poorly infected or few highly infected host cells contribute to disease is still not fully understood. Classic activation of macrophages leads to upregulation of microbicidal activities, which could result in lower intracellular bacterial densities (Grant *et al.,* 2009). However, highly or weakly infected cells apparently expressed similar levels of macrophage activation markers, suggesting that there is no difference in the activation status of these two populations (Thöne *et al.,* 2007). Interestingly, the SPI-2 T3SS of *S.* Typhimurium has been implicated in the downregulation of macrophage activities, by inducing IL-10 (Uchiya *et al.*, 2004), cyclooxygenase-2 (Uchiya and Nikai, 2004) and suppressor of cytokine signalling-3 (Uchiya and Nikai, 2005), which could promote intracellular bacterial replication. Indeed, another intracellular pathogen, *Francisella tularensis,* is also known to induce such responses to facilitate intracellular growth (Shirey *et al.*, 2008). Therefore, it would be interesting to determine if

the activities of highly infected cells observed in *S.* Typhimurium infections might be similarly suppressed.

5. Significance of non-replicating bacteria

Fluorescence dilution also provided information about the replication diversity within a bacterial population. Populations of *S.* Typhimurium recovered from murine tissues were found to contain cells that had undergone different numbers of divisions (Tables 5.3 and 5.6). This could have resulted from differences in the initiation of replication, the replication rate and possibly, the dissemination of bacteria from one tissue to another. Indeed, it has been shown that bacterial populations are inherently heterogeneous, for example with respect to virulence factor expression (Roe *et al*., 2003, Roe *et al*., 2004) or resistance to antibiotic treatments (Balaban *et al*., 2004). Heterogeneous profiles of bacterial replication were also observed within bone marrow-derived macrophages (Helaine *et al.,* 2010). In contrast, we observed that *S.* Typhimurium replicated uniformly within more permissive RAW264.7 macrophages (Fig. 3.6C), demonstrating that bacteria are less stressed within these cells.

Surprisingly, this technique also revealed that, while a population of bacteria replicated inside both macrophages and epithelial cells, a sub-population of bacteria persisted throughout infection without replicating. Further examination of these bacteria in macrophages revealed that they remained within vacuoles (Helaine *et al*., 2010). It is very likely that persistent, non-replicating bacteria have biological significance. Further experiments by our group demonstrated the presence of a large sub-population of intracellular *S.* Typhimurium in splenic macrophages that persisted for several days without undergoing a single division (Helaine *et al.,* 2010). Consistent with this, experiments reported in Chapter 5 revealed that small populations of bacteria that had not replicated were frequently detected within the SI, PP, MLN and spleen at different time-points, for up to at least 2 days following oral inoculation of mice with *S.* Typhimurium (Table 5.1). Further characterisation of intracellular, non-replicating *S.* Typhimurium recovered from infected

splenocytes demonstrated that the majority of these bacteria were able to respond to the presence of an extracellular signal, which was detected by the *de novo* production of DsRed (Helaine *et al.,* 2010). Therefore, intracellular, non-replicating bacteria are metabolically active and still viable.

The presence of non-replicating bacteria *in vivo* is intriguing. It is possible that these bacteria might represent reservoirs that persist in chronic infections, since long-term persistence is thought to be associated with a shut-down in replication and metabolic activity, possibly enabling them to avoid killing by immune defences and antibiotics. Indeed, *S. enterica* is well known to cause chronic asymptomatic and relapsing infections (Bhan *et al.*, 2005). Little is known about how these organisms persist without being killed or where persistent organisms are located. Potential reservoirs of latent bacteria have been proposed in other work, including the PP (Griffin *et al.* 2011), the MLN (Monack *et al.*, 2004a, Griffin *et al.*, 2011), the liver (Nath *et al*., 2010) and the gallbladder (Sinnott and Teall, 1987), perhaps by forming biofilms on existing gallstones (Crawford *et al.*, 2010).

Using fluorescence dilution to identify non-replicating populations of *S.* Typhimurium after oral infection of Slc11a1-resistant mice, which are used to model chronic *S. enterica* infections (Monack *et al.,* 2004a), may help to identify reservoirs of dormant bacteria. Interestingly, Griffin and colleagues have recently developed a murine model of typhoid relapse. In this model, Slc11a1-susceptible mice appear to resolve a primary infection with *S.* Typhimurium, after prolonged treatment with the fluoroquinolone, ciprofloxacin (Griffin *et al.*, 2009, Griffin *et al.*, 2011). However, several days following antibiotic withdrawal, mice develop relapsing disease, most likely from growth initiated within the MLN (Griffin *et al.,* 2011). It would be very interesting to apply fluorescence dilution to study the replication dynamics of *S.* Typhimurium in this system, to determine how non-replicating bacteria respond to antibiotic therapy and how replication dynamics compare between a primary and relapsing infection.

Very recent work by our group has shown that a proportion of non-replicating bacteria resume growth after release from bone marrow-derived macrophages and inoculation in rich laboratory medium or even after uptake in more permissive macrophages (Dr. S. Helaine, personal communication). However, it is still not understood what causes bacteria to enter this non-replicating state. Non-replicating *S.* Typhimurium are not observed in the inoculum, whereas they are detected within host cells and in mice (Helaine *et al.,* 2010 and this study). This suggests that a signal from host cells is sensed by *S.* Typhimurium, which causes bacteria to stop replicating. The nature of this signal remains elusive, as the occurrence of intracellular non-replicating bacteria is independent of the host cell type (Fig. 3.6C and 3.10D) and the bacteriocidal activity of the host cell (Helaine *et al.,* 2010). Interestingly, work described in Chapter 5 of this study provided some insights into the source of the signal sensed by *S.* Typhimurium that might regulate the formation of non-replicating bacteria *in vivo*. In contrast with the presence of non-replicating bacteria in mice after oral (this study) or i.p. inoculation (Helaine *et al.,* 2010), in animals that were inoculated intravenously, all bacteria detected in the spleen had begun to replicate soon after inoculation. These findings suggest that a proportion of *S.* Typhimurium may perceive a signal to repress division within the gastrointestinal tract or peritoneal cavity. Alternatively, the signal to shut-down replication may be actively repressed by some factor sensed in the blood or spleen. Indeed, many reports have shown that bacteria can sense and respond to signals present within the host. Through the histidine kinases, QseC and QseE, *Enterohaemorrhagic E. coli* can sense autoinducer (AI)-3, produced by gut microbial flora, and the mammalian hormones, adrenaline and noradrenaline (Sperandio *et al.*, 2003, Clarke *et al.*, 2006, Reading *et al.*, 2009). These signals lead to upregulation of virulence genes involved in motility, intestinal colonisation and toxin production (Hughes *et al.*, 2009) and can also stimulate bacterial growth (Lyte and Ernst, 1992). A *qseC* homologue is present in *S.* Typhimurium and could also regulate virulence gene expression in response to mammalian catecholamines (Rasko *et al.*, 2008, Merighi *et al.*, 2009, Moreira *et al.*, 2010). Additionally, in one study that used a bovine enteritis model, the administration of noradrenaline was associated with increased bacterial replication (Pullinger *et al.*, 2010). However, the role

of QseC and catecholamine-induced responses in *S.* Typhimurium is disputed (Pullinger *et al.,* 2010, Merighi *et al*., 2009). Nevertheless, it would be interesting to investigate whether the development of a non-replicating phenotype in *S.* Typhimurium may also be influenced by these hormones or bacterial AI-3. Isolating non-replicating bacteria from infected cells or tissues by FACS and comparing their transcriptomes with those that have replicated could provide additional insights into the signals and genes that regulate this phenomenon. Alternatively, screens could be carried out to search for mutants that have an altered frequency of non-replicating bacteria.

6. Conclusions

This thesis describes the development of fluorescence dilution as a technique to measure bacterial replication directly at both the population and single cell level *in vivo*. This work demonstrated that replication is not a prerequisite for *S.* Typhimurium to cross the intestinal wall and seed systemic sites during murine salmonellosis. In fact, the MLN were preferentially seeded by non-replicating bacteria and in contrast, the spleen was preferentially seeded by bacteria that had already replicated. Furthermore, these experiments revealed that the replication of *S.* Typhimurium was more restricted in the MLN compared to the PP, which contributes to an increasing body of evidence that identifies the MLN as a restrictive niche for *S.* Typhimurium during systemic disease. Fluorescence dilution also revealed heterogeneous replication behaviour of *S.* Typhimurium within different environments *in vitro* and *in vivo*. Often a spectrum of replication was observed within a bacterial population, consisting of those that had not replicated or those that had undergone different numbers of divisions. Non-replicating bacteria could represent reservoirs that contribute to chronic and/or relapsing infections. Importantly, the differential fluorescence signal of non-replicating bacteria provides a means to identify and isolate these bacteria from infected cells. Many other bacterial pathogens are also associated with chronic infections, including *Mycobacterium tuberculosis, H pylori*, *Brucella* and uropathogenic *E. coli* (Orme, 2001, Schilling *et al.*, 2002, Monack *et al.*, 2004b, Ehlers, 2009). Applying fluorescence dilution to study bacterial replication dynamics in

models of these infections could provide further insights into the pathogenesis of chronic disease. Lastly, to investigate bacterial organ colonisation in more detail, information about bacterial replication during the evolution of individual infection foci could be obtained by integrating fluorescence dilution with high-resolution imaging, such as intravital multiphoton microscopy (Liu *et al.*, 2007, Månsson *et al.*, 2007). In conclusion, fluorescence dilution provides a means to monitor bacterial replication directly *in vivo,* and therefore can provide more detailed insights into the replication dynamics of bacteria during disease pathogenesis.

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