Characterisation of *Salmonella enterica* serovar Typhi pathogenesis in Human like macrophages

A Thesis submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Date: March 2024

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From May 2023 to March 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled **Characterisation of *Salmonella enterica* serovar Typhi pathogenesis in Human like macrophages** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Soham Vishwas Deolankar at University of Texas Medical Branch at Galveston under the supervision of Prof. Linda J Kenney, Department of Biochemistry & Molecular Biology, University of Texas Medical Branch at Galveston during the academic year 2023-2024.

Linda J Kenney

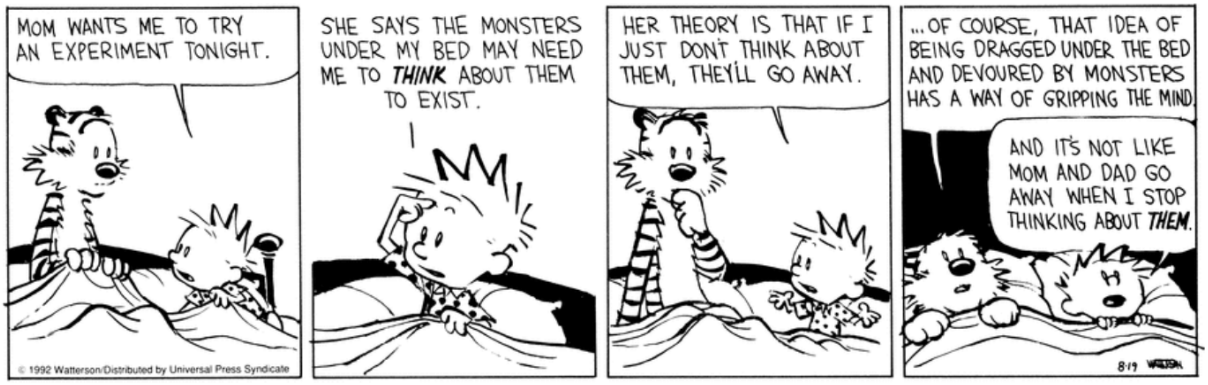
Prof. Linda J Kenney

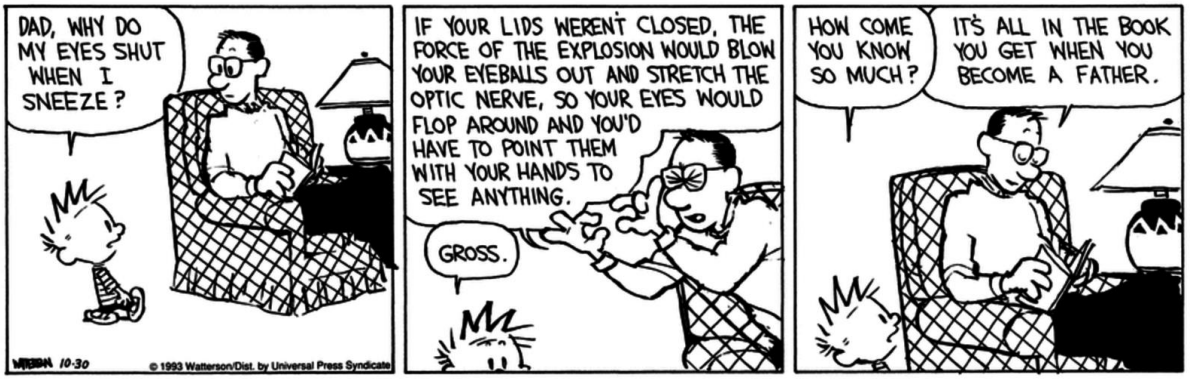
Committee:

Prof. Linda J Kenney

Dr. Nishad Matange

This Thesis is dedicated to my brother and my parents who always had my back while I pushed to test my assumptions at my convenience.





Declaration

I hereby declare that the matter embodied in the report entitled "**Characterisation of *Salmonella enterica* serovar Typhi pathogenesis in Human like macrophages"** are the results of the work carried out by me at the Department of Biochemistry and Molecular Biology, University of Texas Medical Branch at Galveston, under the supervision of Prof. Linda J Kenney, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Soham Deolankar

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Date: 25/03/2024

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Abstract

Salmonella enterica is a common foodborne pathogen with diverse serovars exhibiting different host specificities and disease manifestations. The molecular mechanisms of *Salmonella* pathogenesis have been extensively studied in the broad host range serovar Typhimurium (STm), but less is known about the human-restricted serovar Typhi (STy), responsible for enteric fever. During infection, the pathogen can reside either in a specialised acidic vacuole called the *Salmonella*-containing vacuole (SCV) or the host cytosol. Previous studies from the lab have described the role of cytoplasmic acidification of STm in response to the acidic environment of the SCV. The lowering of internal pH is an essential trigger for downstream processes such as the SsrB-mediated activation of *Salmonella* pathogenicity island-2 (SPI-2) genes, essential for intracellular survival and replication of STm. In this study, we aim to describe the intracellular lifestyle and the mechanisms employed by STy using a clinically relevant strain, H58, for successful infection of human macrophages. We show that STy primarily resides within the SCV during macrophagic infections. Using a novel pH detection probe, mCherryTYG, we demonstrate the cytoplasmic acidification of STy in response to the external acidic stress in the macrophage SCV. Further, we validate the acid-dependent expression of SPI-2 genes by SsrB. Finally, using TIMER^{bac}, we show that the loss of *ssrB* leads to high intracellular replication rates, which results in elevated host cell death. Through our observations, we propose a non-canonical role of SsrB for maintaining persistent infection of macrophages by STy, a hallmark of enteric fever.

Acknowledgments

First and foremost, I would like to thank my supervisor, Prof. Linda J Kenney, for giving me this opportunity to gain her support and guidance. I am grateful for the direct supervision of Dr. Marion Fernandez throughout the project, helping me familiarise myself with the techniques. Her patience and efforts in training me were of the essence during this project. I thoroughly enjoyed the discussions we had as we navigated our way through the project. Thanks to her, I was able to greatly expand my skillset in this relatively short period. I would also like to thank Dr. Nishad Matange for his insights and advice. I am thankful to my lab mates for their stimulating discussions and the opportunity to learn from their expertise. Their company did not allow me to miss home. I would also like to thank all the support staff at UTMB for maintaining the facilities that allowed conducting this comprehensive study.

I would also like to thank all the new friends at Galveston who made this small city feel familiar. Outside of the lab, playing Ultimate Frisbee was something I looked forward to each week; their friendliness and helping nature are something I will carry everywhere I go. I also enjoyed the company of my roommate, Devin Reddy, who was of immense help right from the start and kept my lifestyle in check.

Special thanks to the Kasukabe Defence Group, whose company I always crave in good and bad times. All my friends from IISER Pune who were part of the most memorable memories from the last 5 years. Finally, I would like to thank my parents and brother, who despite their many troubles, always lend me a supporting hand.

Contributions

Contributor name	Contributor role
Soham Deolankar; Dr. Marion Fernandez	Conceptualization Ideas
Soham Deolankar; Dr. Marion Fernandez	Methodology
Dr. Kiran Singh	Software
Soham Deolankar; Dr. Marion Fernandez	Validation
Soham Deolankar; Dr. Marion Fernandez	Formal analysis
Soham Deolankar; Dr. Marion Fernandez	Investigation
Prof. Linda J Kenney	Resources
Soham Deolankar; Dr. Marion Fernandez	Data Curation
Soham Deolankar	Writing - original draft preparation
Soham Deolankar; Prof. Linda J Kenney	Writing - review and editing
Soham Deolankar; Dr. Marion Fernandez	Visualisation
Soham Deolankar; Dr. Marion Fernandez; Prof. Linda J Kenney	Supervision
Soham Deolankar; Dr. Marion Fernandez; Prof. Linda J Kenney	Project administration
Prof. Linda J Kenney	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

1. Introductions

1.1 Introduction to Typhoidal *Salmonella*:

Salmonella enterica is a gram-negative bacterial pathogen, exhibiting the capability to infect a diverse range of hosts. This subspecies is further categorised into serovars by Kauffmann-White classification based on O (lipopolysaccharide) and H (flagellar) antigens (Swaminathan *et al.*, 2000). Depending on the infecting serovar, *Salmonella* can induce various disease states in humans. Broadly *Salmonella* serovars can be categorised into two groups based on the resulting disease states: Typhoidal *Salmonella*, responsible for causing enteric fever, and Non-typhoidal *Salmonella*, known for causing gastroenteritis (Suez *et al.*, 2013). Non-typhoidal *Salmonella* serovars such as Typhimurium (STm) are generalist pathogens with the ability to infect a wide range of hosts, while Typhoidal serovars, like Typhi are human restricted. Among these, *S. enterica* serovar Typhi (STy) stands out as the major causative agent of enteric fever.

Prior to the widespread use of antibiotics half of all reported cases of enteric fever resulted in serious complications and carried a mortality rate over 10% (Stuart and Pullen, 1946). In the year 2019 alone, the World health organisation has reported approximately 9 million cases and 110,000 deaths worldwide by enteric fever, with the highest incidences in regions of south-east Asia, eastern Mediterranean and Africa (Figure 1) (Hancuh, 2023; Meiring *et al.*, 2023).

Recently the emergence of multiple drug-resistant strains of STy has become a significant global health concern. Of particular concern is the widespread dissemination of the STy haplotype H58, already dominant in Southeast Asia and sub-Saharan Africa. Its association with recent typhoid outbreaks and epidemics in these regions raises considerable alarm (Feasey *et al.*, 2015; Pham Thanh *et al.*, 2016). As STy continues to pose a significant threat to global health, there is a pressing need for a comprehensive understanding of its pathogenic mechanisms and host adaptation strategies for these strains.

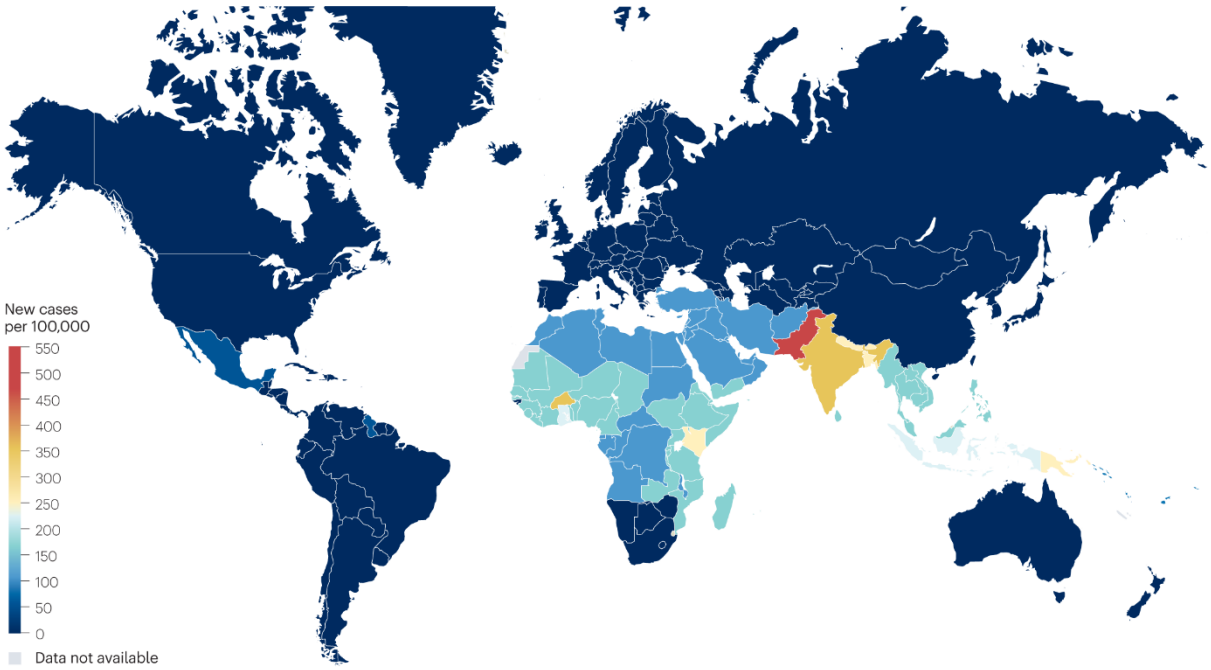

Figure 1. The geographical distribution of enteric fever: Incidence per 100,000 population, 2019 (Plotted using GBD compared by Institute of Health Metrics and Evaluation).

1.2 *Salmonella* pathogenesis:

Salmonella pathogenesis is intricately tied to multiple *Salmonella* pathogenicity islands (SPI) within its genome. To date, 23 SPIs have been reported across the species (Zhao *et al.*, 2020). Phylogenetic analysis has revealed that these islands were horizontally acquired from diverse sources at different points in time (Groisman and Ochman, 1997). These genomic regions harbour crucial virulence genes essential for the survival and proliferation of *Salmonella* within the host cells.

Among these, SPI-1 and SPI-2 assume paramount importance for successful invasion and proliferation of the pathogen. They encode two distinct Type III secretion systems (T3SS), facilitating the transport of virulence factors from the pathogen into the host cytoplasm. Extensive studies of STm have elucidated the functions of SPI-1 and SPI-2 in pathogenesis. SPI-1, with its T3SS and effectors, is vital for catalysing host cell invasion through induced micropinocytosis (Francis et al., 1993) and triggering the host inflammatory response (Galán and Curtiss, 1989; Zhou and Galán, 2001). Conversely, SPI-2 is induced intracellularly, post-invasion and plays a crucial

role in subsequent events of intracellular survival and replication of the pathogen (Ochman et al., 1996; Hensel et al., 1998).

While both STm and STy harbour SPI-1 and SPI-2, the host-directed evolution of STy has led to the functional inactivation of multiple genes, forming pseudogenes within virulence-associated regions (Parkhill *et al.*, 2001; Dagan *et al.*, 2006). Despite the presence of several pseudogenes, STy retains a functional SPI-2 T3SS system (Faucher *et al.*, 2006; Baker and Dougan, 2007). Recent studies have also demonstrated that the SPI-2 encoded T3SS in STy effectively translocate bacterial effector proteins into host cells during infection (Reuter *et al.*, 2021). Nevertheless, the significance of SPI-2 for STy survival in macrophages remains a subject of debate. To date, only one study has documented a replication defect for STy SPI-2 mutants, specifically gene deletions of *ssaV* and *sifA* (Hamblin *et al.*, 2023). Interestingly, although SsrB is well established as an essential regulator of SPI-2 expression in STm (Feng et al., 2003), its deletion in STy exhibited no replication defect (Forest et al., 2010). Similar observations were reported by Reuter et. al. where they compared STy and STm lifestyles in various host cell models (Reuter *et al.*, 2021). Notably, Forest et al. and Reuter et al. employed a gentamicin protection assay followed by enumeration of colony-forming units (CFU) as a measure of intracellular replication rate, while Hamblin et al. utilized an arabinose-inducible fluorescence dilution method for mapping intracellular replication rates. These contradictory results raise the intriguing possibility that the absence of a phenotypes observed in some studies may be an artifact induced by ensemble-based assays such as the gentamicin protection assay. Further investigations could reconcile these discrepancies and gain a more nuanced understanding of the role of SPI-2 in STy pathogenesis within macrophages.

1.3 Entry and infection:

Salmonella is typically acquired by ingestion of contaminated food or water with the infectious dosage described for STm to be $10^3 – 10^5$ (Blaser and Newman, 1982). Following ingestion, bacteria surviving the highly acidic environment of the stomach then reach the small intestine, where it catalyses uptake by the epithelial cells of the mucosa (Haraga *et al.*, 2008). This invasion by non-phagocytic cells is mediated by a type three secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI-1) (Francis *et al.*, 1993; Garcia-del Portillo and Finlay, 1994). After crossing the

epithelial barrier of the intestinal mucosa, *Salmonellae* can then be internalised by the subepithelial macrophages (Alpuche-Aranda *et al.*, 1994) (Figure 2). The invasion of macrophages by *Salmonella* marks the onset of systemic disease or enteric fever. While STm infection is usually limited to gastrointestinal regions, STy has been documented to show higher persistence in macrophage infection, reaching the spleen and lymph nodes.

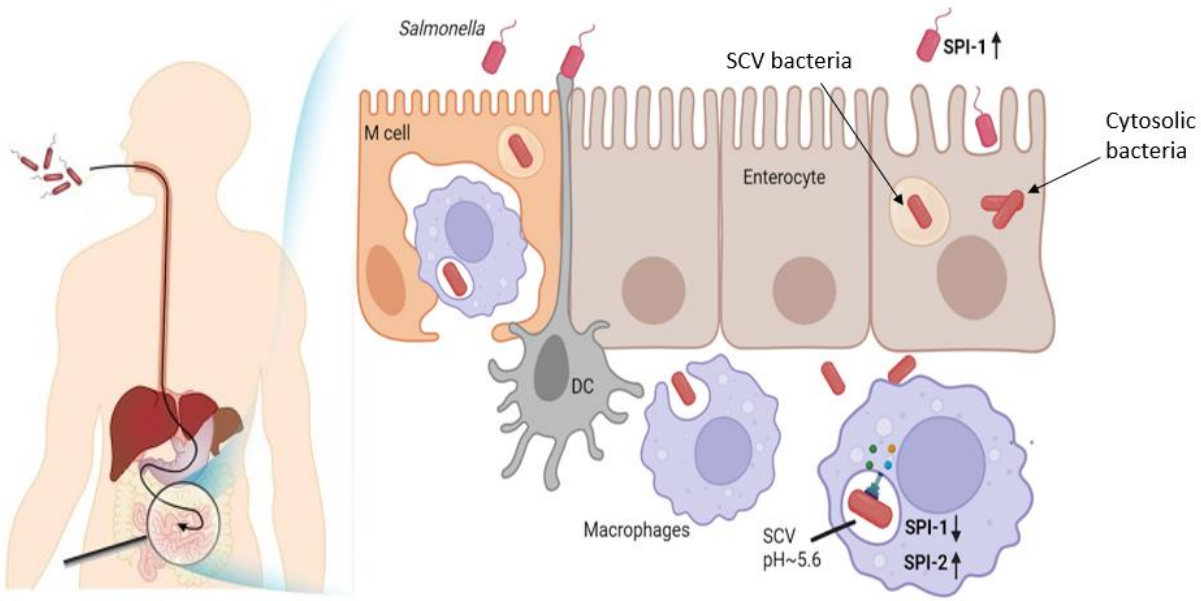

Figure 2. Acquiring *Salmonellae*: Orally ingested *Salmonellae*, after surviving the acidity of the stomach, reach the small intestine. Here, they infect the epithelium by SPI-1 mediated invasion. Crossing the mucosal epithelium, bacteria can be acquired by macrophages resulting in systemic infection.

Once internalised, *Salmonella* resides inside specialised phagosomes known as the *Salmonella* containing vacuole (SCV) (Alpuche-Aranda *et al.*, 1994). As the infection progresses. the SCV further matures by fusing with multiple lysosomes and acidifies its internal environment (Buchmeier and Heffron, 1991; Oh *et al.*, 1996) (Figure 3). This low pH serves as an essential trigger for activation of SPI-2 genes in STm, essential for intracellular survival and proliferation (Alpuche Aranda *et al.*, 1992; Chakraborty *et al.*, 2015; Liew *et al.*, 2019). While the SCV serves as the primary niche

for bacterial replication, some bacteria can escape into the host cytosol (Steele-Mortimer et al., 2010).

The SCV and the cytosol of the host cell provide very different environments for bacteria replication such as high osmotic and acid stress in the SCV, while the availability of nutrients in the cytosol is higher (Steele-Mortimer *et al.*, 2012; Liss *et al.*, 2017). Multiple studies have described differential lifestyles for STm in various host types and its localisation within the host cell. In HeLa cells, STm has been documented to show a hyper replicative phenotype, which accounts for the majority of intracellular replication (Steele-Mortimer *et al.*, 2012). In another instance, a dormant STm population have been described in HeLa cells residing in specialised vacuoles (Luk *et al.*, 2021). Although the epithelial cell cytoplasm supports robust intracellular growth in epithelial cells, *Salmonella* do not fare as well in macrophages and fibroblasts (Beuzón *et al.*, 2002).

While studies on STm have provided essential insights on *Salmonella* intracellular lifestyles, similar studies characterising STy have not been performed. Moreover, one of the hallmarks of enteric fever is the persistent infection of macrophages, and thus highlights the need for characterising and quantifying the intracellular populations of STy in macrophages.

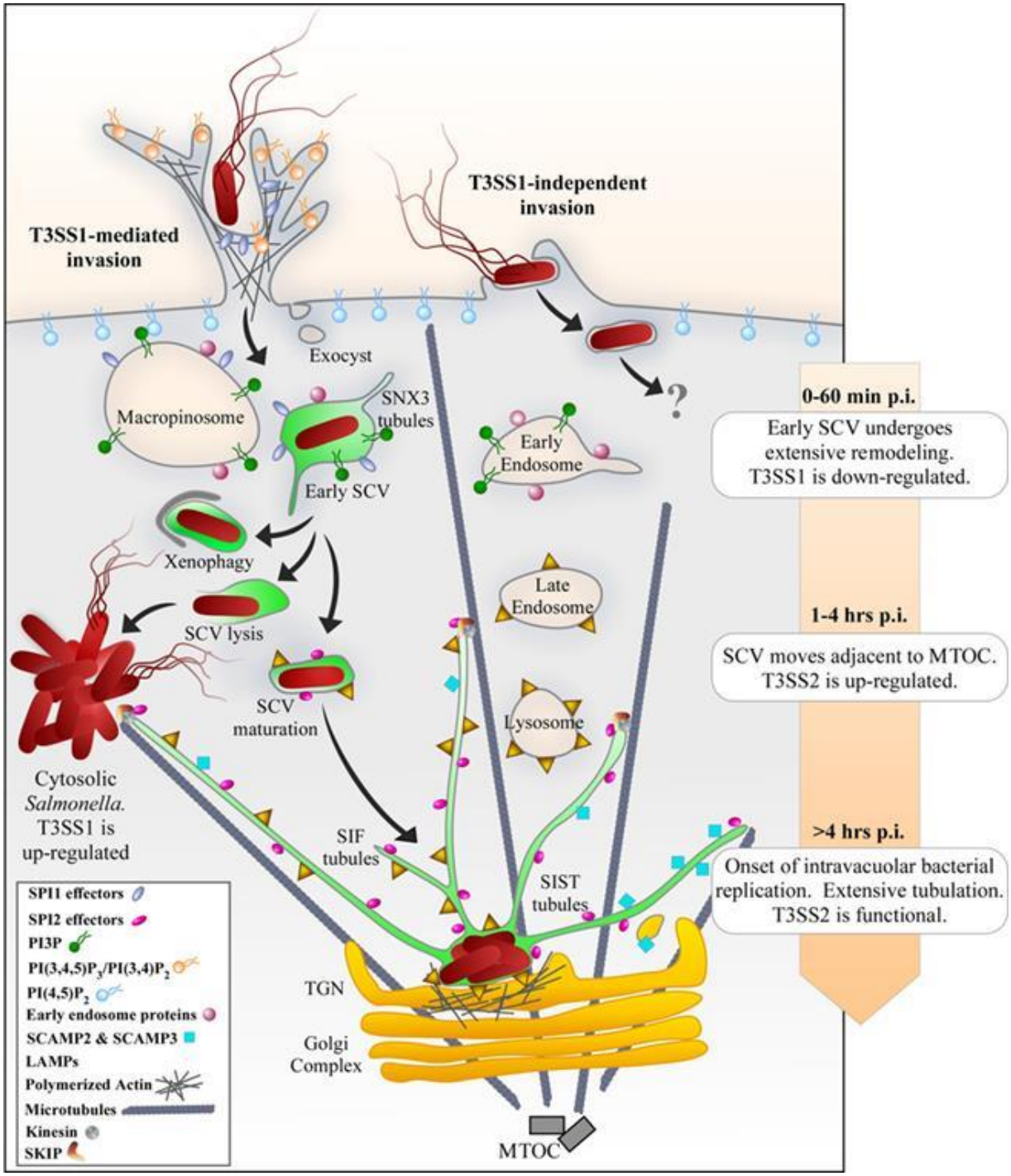

Figure 3. *Salmonella* intracellular lifestyle in host cell and SCV biogenesis: Multiple protein markers are associated with the SCV membrane at various stages. *Salmonella* shows a distinct lifestyle based on its localisation inside the host cell. (Reproduced from Malik-Kale et al).

1.4 The acid response in STm:

Prior studies have demonstrated that the SCV, upon fusing with lysosomes during maturation can become as acidic as pH 5 (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996). To survive within this highly acidic environment of the SCV, *Salmonella* employs a diverse array of adaptations, including the regulation and maintenance of the SCV environment and the acidification of its own cytosol in response to the acidic environment (Liew *et al.*, 2019). The sensitivity of *Salmonella* to external pH underscores the importance of monitoring intracellular pH changes, as it provides valuable insights into the sequential responses exhibited by the pathogen within the host. Multiple studies have employed various pH tracking probes to study the intracellular pH changes. A comparison of these pH sensing probes is provided in the appendix for reference.

Previous studies from our lab have elucidated the underlying mechanism and emphasised the importance of cytoplasmic acidification in STm for intracellular survival. This acid stress response is mediated by the OmpR/EnvZ two-component system, where EnvZ acts as the sensor kinase activating OmpR (Chakraborty *et al.*, 2015, 2017). The activation of OmpR represses the *cadC/BA* operon, encoding the lysine decarboxylation pathway, which is normally responsible for recovery from acid stress by proton consumption. Inhibition leads to acidification of the cytosol (Chakraborty *et al.*, 2017).

This cytoplasmic acidification also serves as an essential trigger for multiple downstream processes. The phosphorylation of OmpR by EnvZ simulates the activation of the *ssrA/ssrB* two-component system (Feng *et al.*, 2003). This, along with the activation of the PhoP/Q two-component system, boosts *ssrB* transcription even more so than the EnvZ/OmpR mediated activation (Bijlsma and Groisman, 2005; Liew *et al.*, 2019). SsrB directly oversees the activation of SPI-2 genes by binding upstream of promoter sequences and facilitating RNA polymerase recruitment while concurrently displacing H-NS, a nucleoid-structuring protein and a global transcriptional repressor in *Salmonella* (Walthers *et al.*, 2011). A recent publication from our lab has shown that the acidic pH enhances the DNA binding ability of SsrB (Liew *et al.*, 2019; Shetty and Kenney, 2023). Thus, linking the role of SsrB-mediated SPI-2 activation with cytoplasmic acidification.

While the phenomenon of cytosol acidification in STm has been thoroughly characterised, shedding light on its pivotal role in intracellular survival, a comparable investigation in STy has not been performed.

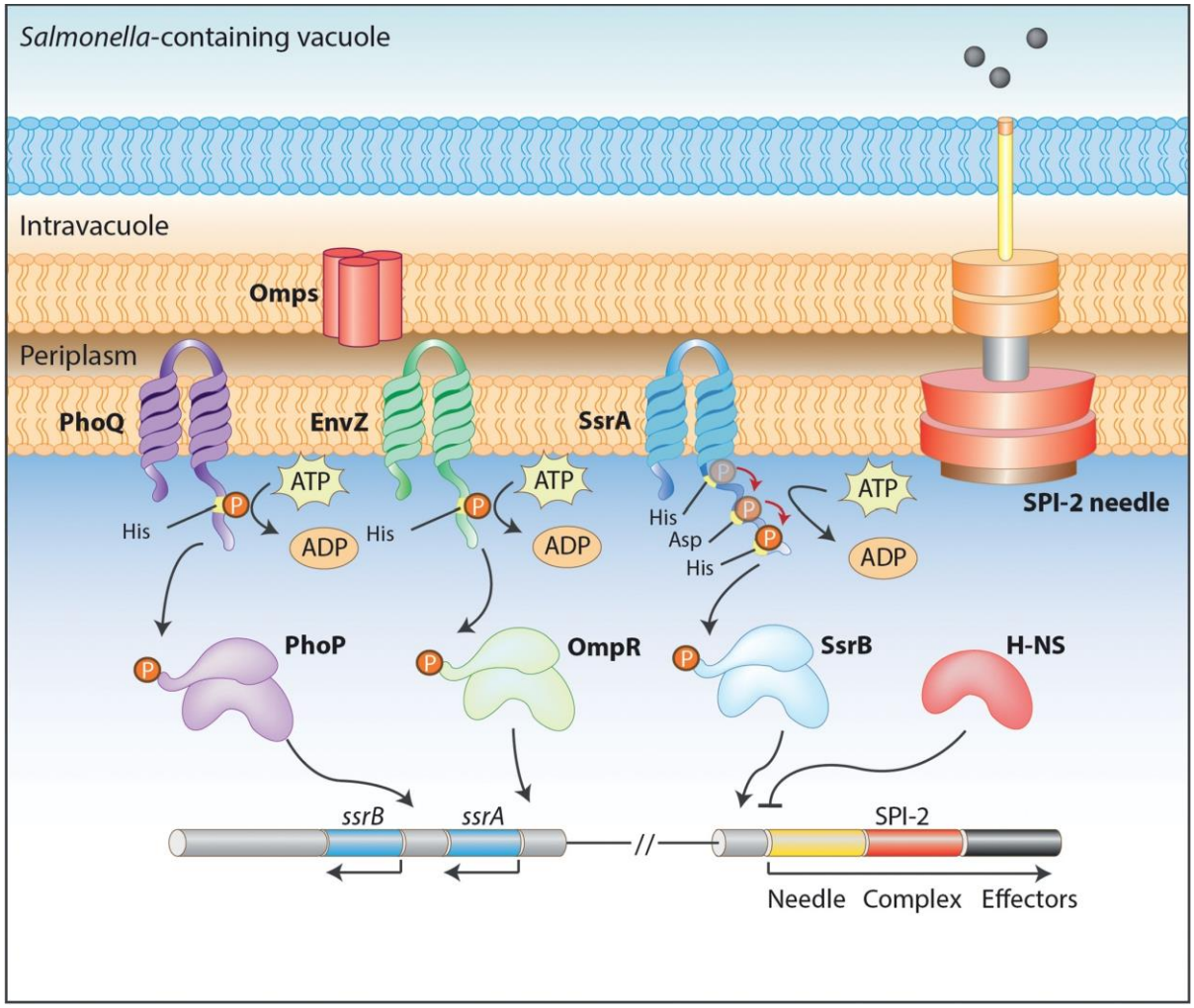

Figure 4: SPI-2 regulation by two-component systems: EnvZ-OmpR activates *ssrA* and *ssrB* expression, PhoQ/P boosts *ssrB* expression. SsrB in turn activates SPI-2 gene expression by relieving H-NS silencing. (Adapted from Kenney, 2019)

1.5 Scope of this Study:

Although recent studies have delved into the molecular mechanisms of STy pathogenesis across various host cell types, a comprehensive understanding of host-pathogen interactions remains elusive. For instance, while some studies have extrapolated mechanistic models developed for STm to STy, critical pathogenic events such as cytoplasmic acidification in response to external acid stress and the regulatory

role of SsrB in SPI-2 expression in STy have not been adequately explored. Our study aims to address these knowledge gaps and further elucidate a novel role of SsrB in STy pathogenesis.

Our findings reveal that STy predominantly localizes within the SCV throughout the infection, showcasing its adaptability to this intracellular niche. Additionally, our novel in-house developed pH sensing probe demonstrates that STy acidifies its own cytosol in response to the acidic stress within the SCV. These results were further corroborated *in vitro*, highlighting the relevance of the acid stress response *in vivo*.

Moreover, we validate the SsrB-mediated expression of the SPI-2-associated gene *sifA* in STy, emphasizing the regulatory role of SsrB in virulence gene expression. Importantly, we confirm the essential role of cytoplasmic acidification in mediating SsrB-dependent SPI-2 gene expression.

While our findings align STy behaviour with the established model developed using STm, our investigation into the role of SsrB for intracellular survival and replication yields novel insights. Specifically, we observe a significantly heightened replication rate of STy in *ssrB* null backgrounds, which correlates with increased host cell death compared to WT strains. These observations suggest a novel role of SsrB in maintaining persistent infection of STy in macrophages, a hallmark of enteric fever.

2. Materials and Methods

Bacterial strains and culture conditions:

Salmonella enterica serovar Typhi strain H58 was used as the Wild Type strain. Bacterial cultures were grown in Luria-Bertani Broth (LB) media supplemented with 100 µg/mL ampicillin, 25 µg/mL chloramphenicol or 12.5 µg/mL tetracycline when required at 37°C with shaking at 200 RPM. All bacterial strains are described in Table 1.

Cell culture and differentiation:

THP-1 acute monocytic leukaemia cells were purchased from ATCC. THP-1 cells were routinely cultured in Roswell Park Memorial Institute 1640 Media (RPMI-1640) complete media (Refer to Table 2), incubated at 37°C, 5% CO_2 and 80% humidity. Prior to infection, THP-1 cells were seeded in 24 well plates at a density of $1x10^5$ cells per well and differentiated into macrophage-like cells by treating with 10 uM PMA (phorbol 12-myristate 13-acetate) for two days.

Table 1: List of bacterial strains used in this study		
WT H58	Wild type *Salmonella enterica* serovar Typhi H58	Lab Strain
ssrB::Cm^R	*ssrB* replaced with Chloramphenicol resistance cassette in WT H58	This Work
WT mCherry	WT H58 harbouring pFPV mCherry plasmid	This Work
ssrB::Cm^R mCherry	H58 *ssrB*::Cm^R harbouring pFPV mCherry plasmid	This Work
WT mCherryTYG	H58 WT harbouring mCherryTYG pH probe expression plasmid	This Work
ssrB::Cm^R mCherryTYG	*ssrB*::Cm harbouring mCherryTYG pH probe expression plasmid	This Work

WT TIMER	H58 WT harbouring the TIMER^{bac} module carrying plasmid	This Work
ssrB::Cm^{R} TIMER	*ssrB*::Cm harbouring the TIMER^{bac} module carrying plasmid	This Work
WT PsifA-tagBFP	H58 WT harbouring the *PsifA* activity reporter plasmid	This Work
WT cadBA PsifA-tagBFP	H58 WT harbouring the *PsifA* activity reporter plasmid and pBR322 Pamp-cadBA	This Work
WT pZS mCherry	WT H58 harbouring pZS mCherry plasmid for setting BFP signal threshold	This Work
ssrB::Cm^{R} PsifA-tagBFP	*ssrB*::Cm harbouring the *PsifA* activity reporter plasmid	This Work

Table 2: List of Plasmids used in this study

Strain/Plasmid	Description	Source
pKD3	Plasmid encoding frt-flanked chloramphenicol cassette	(Datsenko and Wanner, 2000)
pKD46	Plasmid carrying λ red genes under arabinose inducible promoter, temperature-sensitive (grown at 30°C for plasmid maintenance)	
pCP20	Plasmid encoding FLP recombinase and ampicillin resistance, temperature-sensitive (grown at 30°C for plasmid maintenance)	
pBR322 TIMER^{bac}	Plasmid encoding the TIMER^{bac} module under the constitutive *ybaJp* promoter.	Addgene (plasmid # 103056)

pFPV mCherry	Plasmid encoding the mCherry fluorophore under the constitutive *rpsM* promoter.	Lab Stock
pFPV mCherryTYG	Plasmid encoding the mCherryTYG pH probe under the constitutive *rpsM* promoter.	Lab stock (Dr. Kiran Singh)
pZS mCherry	Plasmid with constitutive mCherry expression under *tet* promoter	Lab stock
pZS mCherry PsifA-tagBFP	pZS mCherry plasmid encoding the BFP fluorophore under the *sifA* promoter for checking *sifA* promoter activity.	This Work
pBR322 Pamp-cadBA	Plasmid used for overexpression of the *cadBA* for prevention of cytoplasmic acidification	Lab stock (Dr. Chakraborty)
pLenti LAMP1-miRFP703 Puro	Entry vector carrying the LAMP1-miRFP670 fusion for transfection of THP-1 cell line	This Work
pMDL	3rd generation lentiviral packaging plasmid containing gag and pol.	Gracious gift from Dr. Subramanian
pRSV	3rd generation lentiviral packaging plasmid that encoding rev.	
pVSV	Lentiviral packaging plasmid encoding the VSV envelope protein.	

Table 3: Media and Buffers with compositions

Buffer/Media	Composition
Modified N-minimal medium (MgM)	7.5mM Ammonium sulphate 7.5mM Potassium Chloride 0.5mM Potassium Sulphate

	1mM Monopotassium phosphate 10uM Magnesium Chloride 2mM Glucose 0.1% Casamino acids (Sigma) 100mM Tris for pH 7.2 / 100mM MES for pH 5.6 pH adjusted by to required pH using HCl and NaOH
KHM Buffer	110mM Potassium Acetate 20mM HEPES 2mM Magnesium Chloride Adjusted to pH 7.3 using HCl and NaOH
RPMI-1640 complete media	RPMI-1640 medium (ATCC# 30-2001) 25mM HEPES buffer 2mM L-Glutamine 10% (v/v) Heat-inactivated Fetal Bovine serum 1% modified Eagle's medium amino acids solution 1mM Sodium Pyruvate 1% Penicillin + Streptomycin
Luria-Bertani Broth (LB)	2.5% Luria Bertani Broth, Miller (HiMedia ref: GM1245-500G) in MilliQ water. Autoclaved at 15 psi pressure for 30 mins at 121°C.
Luria-Bertani Broth Agar (LB agar)	16% Luria Bertani Agar Broth, Miller (HiMedia ref: GM151-500G) in MilliQ water. Autoclaved at 15 psi pressure for 30 mins at 121°C.

Table 4: Oligonucleotides used

Primer name	Sequence (5' - 3')	Purpose
ssrB_ko_FP (forward)	AGAATATAAGATCTTATTAGTAGACGATCATGAAATCATCGTGTAGGCTGGAGCTGCTTC	*ssrB* knockout in H58 WT by λ-Red recombination
ssrB_ko_RP (reverse)	GTAACTCTGTCACTTTATGAACCTGTAGCTTTCTCATCATCATATGAATATCCTCCTTAG	

ssrB_chk_FP (forward)	ACTTATCCTTGATCTTAGCT	Confirming *ssrB* knockout
ssrB_chk_RP (reverse)	TATGGATTTTGTCGATGATG	
PsifA_FP (forward)	TTATCAGAATCGCAGATCTGTTGATTTACTGGCGGGCT	PCR amplification of *sifA* promoter from H58 WT gDNA for clonning into pZS mCherry
PsifA_RP (reverse)	CGCCCTTAGACACCATATTAATCTCACTTATACTGG	
pZS_FP1 (forward)	CCAGTATAAGTGAGATTAATATGGTGTCTAAGGGCGAAGA	PCR amplification of pZS mCherry plasmid backbone
pZS_RP1 (reverse)	CAATTCTGGAAGAAATAGCGAAAGGCCGCTAAAGCGGCT	
pZS_FP2 (forward)	AGCCCGCCAGTAAATCAACAGATCTGCGATTCTGATAAC	
pZS_RP2 (reverse)	CGGCCTTTCGCTATTTCTTCCAGAATTG	
LAMP1_FP (forward)	TTATCAGAATCGCAGATCTGTTGATTTACTGGCGGGCT	PCR amplification of LAMP1 protein for cloning into pLentiPuro backbone
LAMP1_RP (reverse)	CGCCCTTAGACACCATATTAATCTCACTTATACTGG	
pLentiPuro_FP1 (forward)	CCAGTATAAGTGAGATTAATATGGTGTCTAAGGGCGAAGA	PCR amplification of pLentiPuro backbone for lentiviral transdction of LAMP1-miRFP703
pLentiPuro_RP1 (reverse)	CAATTCTGGAAGAAATAGCGAAAGGCCGCTAAAGCGGCT	
pLentiPuro_FP2 (forward)	AGCCCGCCAGTAAATCAACAGATCTGCGATTCTGATAAC	
pLentiPuro_RP2 (reverse)	CGGCCTTTCGCTATTTCTTCCAGAATTG	

Isolation of Plasmid DNA:

A single bacterial colony was picked and inoculated into 10 mL LB and incubated overnight at 37°C with shaking at 200 RPM. For plasmid isolation, the

QIAprep Spin Miniprep Kit (Cat. No. 27106) was used following the protocol provided. Plasmid DNA was eluted in 25 µL warm milliQ water and the concentration was determined by nanodrop.

Agarose Gel Electrophoresis:

1% agarose solution was prepared using 1x TAE (Tris-acetate-EDTA) buffer as the solvent. The solution was boiled and allowed to cool down and solidify in a gel cast tray after supplementing the solution with SYBR Safe. Gel electrophoresis was performed by supplying a voltage of 150 V. A gel loading buffer was added to the samples and the samples were subjected to gel electrophoresis along with a DNA ladder until a sufficient separation was achieved.

Preparation of Heat-shock competent cells:

Cultures were inoculated from single colonies of freshly streaked LB agar plates added to 3 ml LB with antibiotics. Cultures were grown overnight at 37°C with 200 RPM shaking. 1% of the overnight culture was inoculated into 50 mL LB and was grown for 2 hours or until OD 600 ~0.6 at 37°C with 200 RPM shaking. The supernatant was discarded, and the pellet was resuspended in 10 mL cold 0.1M $CaCl_2$. The suspension was then incubated on ice for 30 mins, centrifuged again for 10 mins at 3000 x g at 4°C. The supernatant was discarded, and the pellet was washed two times with 10 mL cold 0.1 M $CaCl_2$. The supernatant was discarded and washed once with 0.1 M $CaCl_2$ dissolved in 10% glycerol. The pellet was then resuspended into 1 mL of 0.1 M $CaCl_2$ dissolved in 10% glycerol. The resulting suspension was aliquoted into microcentrifuge tubes such that each tube had 50 µL of the suspension and stored at -80°C.

Transformation by Heat-shock:

Competent cells and plasmid DNA were thawed on ice. To transform the competent cells, ~400 ng was added to 1.5 mL tube containing 50-60 µL of competent cells. The mixture was incubated on ice for 30 mins and then subjected to a heat shock by submerging the tube in a water bath at 42°C for 60 seconds. The tube was immediately placed back on ice. To allow the cells to recover, 1 mL of LB media was added to the mixture and incubated at 37°C with shaking at 200 RPM for 2 hours. The culture was centrifuged for 1 min at 13000 RPM and resuspended in 100 µL LB. The

resultant suspension was plated on LB agar plates supplemented with the appropriate selection antibiotic. The plate was then incubated at 37°C overnight for colonies.

Preparation of electro-competent cells:

Cultures were inoculated from single colonies of freshly streaked LB agar plates and added to 3 ml LB with antibiotics. Cultures were grown at 37°C with 200 RPM shaking overnight. A 1:30 dilution of overnight culture was reinoculated in 12 ml LB and grown at 37°C with 200 RPM shaking for ~2hrs or until OD 600 ~0.5. The culture was then pelleted by centrifuging at 3000 x g for 10 mins at 4°C. The supernatant was removed, and the pellet was washed twice with 10 mL cold sterile milliQ water. The pellet was washed once with 10% glycerol in milliQ water. The pellet was then resuspended in 600 µL of 10% glycerol solution. The resultant suspension was aliquoted into precooled microcentrifuge tubes such that each tube held 60 µL of the suspension and stored at -80°C.

Transformation by Electroporation:

Competent cells and plasmid DNA were thawed on ice. To transform the competent cells ~400ng of plasmid was added to a microcentrifuge tube containing 50-60 µL of competent cells and the mixture was transferred to 2 mm electroporation cuvettes. Bacteria were electroporated by a 2.5 kV pulse using the Gene Pulser-Xcell System. Transformed cells were recovered in 1 mL of LB media at 37°C with shaking at 200 RPM for 2 hours. The culture was centrifuged for 1 min at 13000 RPM and resuspended in 100 µL LB. The resultant suspension was plated on LB agar plates supplemented with the appropriate selection antibiotic. The plate was then incubated at 37°C overnight for colonies.

Construction of *ssrB* null STy:

The *ssrB*::Cm^R strain was generated by replacing the *ssrB* gene with a chloramphenicol cassette flanked by FRT sites (Cm^R) as described in Datsenko and Wanner, 2000. In short, primers were designed with ~40 bp homology to the *ssrB* gene and 20 bp homology with Cm^R. Cm^R was PCR amplified from pKD3 plasmid and gel purified. Competent cells harbouring the lambda red plasmid (pKD46) were transformed by electroporation using ~400 ng of Cm^R PCR product. Cells were allowed

to recover in LB media for 2 hours at 30°C with shaking and allowed to grow over night on the bench. The culture was plated on LB agar supplemented with 25 µg/mL chloramphenicol for selection. Colonies were checked for replacement using colony PCR with primers overlapping Cm^R.

THP-1 infection:

Bacteria were grown as a standing overnight culture in 3 ml LB media with appropriate antibiotics at 37°C. Bacterial cultures were diluted with infection media complete (RPMI-1640 complete media without antibiotics) to a multiplicity of infection of 1:10. Infection was synchronised by centrifuging plates at 600 x g for 5 mins and then incubated at 37°C, 5% CO_2 for 25 mins. Wells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) containing 100 µg/mL gentamicin x 3 and incubated with infection media with 100 µg/mL gentamicin at 37°C, 5% CO_2 for 30 mins. Infected THP-1 cells were then incubated in infection media containing 12 µg/mL of gentamicin until time points. Time post-infection indicated has been counted from the first DPBS + 100 µg/mL gentamicin washes.

Generation of THP-1 LAMP1-miRFP703 cell line:

A plasmid Lentiviral compatible plasmid expressing a translational fusion of LAMP1 protein and the fluorophore miRFP703 was constructed by Dr. Marion Fernandez (Refer to table 4 for primers). Transfection of the WT THP-1 cell line with the above mentioned plasmid was performed following a protocol developed by the lab of Dr. Naeha Subramanian (Rommereim *et al.*, 2021) with slight changes in viral particle collection from HEK93T cells. Briefly, ~48 hours post transfection of media was collected containing viral particles into a sterile centrifuge tube. Cellecta's LentiFuge™ Viral Concentration Reagent was added to the collected media and left overnight at 4°C. Viral particles were pelleted by centrifuging at 15,000 x g for 1 hour at 4°C. The supernatant was decanted and the pellet resuspended in RPMI-1640 complete media and used immediately for transfection of THP-1 cells following instructions from Rommereim *et al.*, 2021.

Phagosome protection assay:

To quantify the distribution of cytosolic and vacuolar bacterial populations, we used a digitonin permeabilisation assay (Meunier and Broz, 2015). The digitonin concentration for THP-1 cells was optimised such that antibodies recognizing the luminal part of LAMP1 could not tag the protein, indicating an intact SCV (Steele-Mortimer et al., 2014), while ensuring the plasma membrane was permeabilised, as indicated by phalloidin labelling of cytosolic actin. The optimal digitonin concentration was determined to be 15 µg/mL for 1 min. Seeded THP-1 cells were infected with constitutively mCherry-expressing *Salmonella* as described above. At the indicated times, cells were digitonin-treated and cytosolic bacteria were tagged using Anti-LPS antibodies and Anti-Vi capsule antibodies. Post immunostaining, samples were fixed using 4% PFA and cytosolic bacteria were revealed by anti-rabbit Alexa 488 and quantified using ImageXpress Pico Automated Cell imaging system for cell scoring.

Measurement of mCherryTYG fluorescence lifetime (*in vitro*):

Overnight grown cultures of concerned *Salmonella* strains expressing mCherryTYG were subcultured (1:30 dilution) into 3 mL LB media adjusted to pH 7 and pH 4.5 and grown until OD600~ 1 at 37°C with shaking at 200 RPM. 200 µL of culture was aliquoted for each pH and pelleted by centrifuging at 4500 x g for 5 mins. Supernatant was discarded, and the pellet was resuspended in 20 µL of LB of the same pH. 0.5 µL of the resulting suspension was used to prepare samples on pH-adjusted agarose pads (1.5%). The mCherryTYG fluorophore lifetime was recorded for fifty or more bacteria at 580 nm after excitation at 561 nm.

Measurement of mCherryTYG fluorescence lifetime (*in vivo*):

THP-1 cells were seeded in ibidi µ-Slide 8 Well plates (Catalog # 80806, Ibidi) and infected with *Salmonella* expressing mCherryTYG as described above using Phenol red free infection media. At the indicated time points, samples were maintained at 37°C and 5% CO_2 using Live-Cell Imaging Perfusion Chamber and observed under the confocal microscope for recording fluorescence lifetimes. The mCherryTYG lifetime was recorded for fifty or more THP-1 cells harbouring STy at 580 nm after excitation at 561 nm.

Gentamicin protection assay:

To characterise the survival of various mutants compared to WT, we employed a gentamicin protection assay, which uses colony forming units (CFU) from each sample/well as a measure of survival. THP-1 cells were seeded and infected with *Salmonella* strains as described above. At the indicated time points, wells were washed with DPBS, and macrophages were lysed using 0.1% Triton-X at RT for 10 mins. The solution was agitated by pipette mixing to resuspend the released bacteria. 50 µL of supernatant was collected from each well and serially diluted in Phosphate-buffered saline (PBS). Dilutions 10^{-3} were plated on Luria-Bertani agar medium (LA) plates. Plates were incubated overnight at 37°C and CFUs were determined.

Flow cytometry analysis of TIMER^{bac}:

THP-1 macrophages were infected with *Salmonella* strains carrying the plasmid pBR322-TIMER. At 30 mins, 2, 6 and 24 hours p.i. THP-1 cells were lysed using 0.1% Triton X-100 for 10 mins, and released bacteria were collected, washed with cold PBS and fixed using 1.5% PFA for 30 mins. Fixed bacteria were washed with cold PBS to remove excessive PFA and stored at 4°C for flow cytometry analysis. The fluorescence intensities of the samples were analysed with BD FACSymphony A5 SE cell analyser. The green state of TIMER^{bac} was recorded with excitation: 488 nm emission: 510/10 nm; and the red state with excitation: 562 nm emission: 580/10 nm and analysed using FlowJo (v10.0.4). At least 2000 cells were analysed for each strain at each time point.

Construction of reporter plasmid for *sifA* promoter activity:

To accesses *sifA* promoter activity a dual fluorescent protein reporter plasmid was generated (pZS mCherry PsifA-tagBFP) by Dr. Marion Fernandez. *sifA* promoter was PCR amplified from STy WT gDNA using primers PsifA_FP and PsifA_RP. Plasmid backbone was amplified using two set of primers pZS_FP1/RP1 and pZS_FP2/RP2. DNA fragments were PCR purified using gel elution (Qiagen Gel extraction kit) and ligated by Gibson Assembly Cloning Kit.

***SifA* promoter activity assay (*in vitro*):**

Overnight grown cultures of *Salmonella* strains carrying the plasmid pZS mCherry PsifA-tagBFP were sub-cultured (1:30 dilution) into 3 mL LB media adjusted

to pH 7 and 4.5 and grown until OD600 of ~1 at 37°C with shaking at 200 RPM. 200 µL aliquots of culture were pelleted by centrifugation at 4500 x g for 5 mins and resuspended in 20 µL pH-adjusted LB media. 0.5 µL of the resulting suspensions at each pH were used to prepare samples on pH-adjusted agarose pads (1.5%). mCherry fluorescence intensity was recorded at 610 nm post excitation at 580 nm. BFP fluorescence intensity was recorded at 440 nm post excitation at 400 nm. Fifty or more bacteria were quantified per strain at each pH. Analysis was performed using RatioPlus plugin from FIJI ImageJ.

***SifA* promoter activity assay (*in vivo*):**

THP-1 macrophages were infected with *Salmonella* strains carrying the plasmid pZS mCherry PsifA-tagBFP. At 30 mins, 2, 6 and 24 hours p.i. THP-1 macrophages were lysed using 0.1% Triton X-100 for 10 mins and the released bacteria were collected, washed with cold PBS and fixed using 1.5% PFA for 30 mins. Fixed bacteria were washed with cold PBS to remove excessive PFA and stored at 4°C for flow cytometry analysis. BFP and mCherry signals from isolated bacteria were recorded using BD FACSymphony A5 SE cell analyser and analysed with FlowJo (v10.0.4). BFP signal from at least 5000 mCherry positive cells was recorded for each strain at each time point.

3. Results

3.1 STy predominately localise to the SCV in THP-1 macrophages:

Characterising the bacterial localisation is essential to understanding STy pathogenesis due to the contrasting environment offered by the SCV and the cytoplasm of macrophages. Various tools were developed to identify the cytosolic population from the vacuolar population of STy within THP-1 cells.

Initial attempts to visualise the SCV involved immunostaining of LAMP1, a protein associated with the late endosomal membrane and regarded as a marker for SCVs (Drecktrah *et al.*, 2007). However, our analysis of fixed THP-1 macrophages infected with mCherry-expressing STy revealed an unreliable association between LAMP1-labelled membranes and the bacteria. This inconsistency aligns with a prior study highlighting that the fixation of *Salmonella*-infected cells can impair the visualisation of LAMP1-positive SCV membranes (Rajashekar *et al.*, 2014). Live cell imaging presents clear advantages over fixed sample analysis, particularly in preserving membrane structures and enabling the integration with additional techniques.

Given these challenges, our approach shifted towards developing a stable THP-1 cell line expressing a fluorescently-tagged LAMP1. A plasmid expressing a translational fusion of LAMP1 with the fluorophore miRFP703 (pLenti LAMP1-miRFP703 Puro) was constructed and was introduced into WT THP-1 cells using a lentiviral transduction protocol established by the lab of Dr. Naeha Subramanian (Rommereim et al., 2021), which successfully yielded a stable THP-1 cell line expressing LAMP1-miRFP703 (THP-1 LAMP1-miRFP703). Surprisingly, microscopy analysis of this LAMP1-miRFP703-expressing THP-1 cell line revealed patterns similar to those observed in fixed samples, with inconsistent bacterial enclosure by LAMP1-labeled membranes (Figure 5A). This observation resembles recent findings where traditional SCV markers failed to colocalise with vacuolar STm in HeLa cells (Luk et al., 2021), suggesting that the SCV in THP-1 macrophages might not conform to established models. This raises intriguing questions about the nature and behaviour of SCVs in THP-1 cells and potentially points to novel mechanisms of STy pathogenesis within various host cells (see Discussion).

Owing to the complexity of reliably using known SCV markers in THP-1 cells, we employed a phagosome protection assay to quantify vacuolar and cytoplasmic populations in THP-1 cells. The assay relies on selective permeabilisation of the plasma membrane using the non-ionic detergent, digitonin. Once permeabilised, anti-*Salmonella* lipopolysaccharide (LPS) and Anti-Vi capsule antibodies were delivered directly to the cytosol. The number of labelled bacteria were scored using fluorescence microscopy at 2, 6, and 24 hours post infection (p.i.). More than 9000 bacteria were scored for each timepoint spread across three replicates. The percentage of cytosolic bacteria ranged from 15-20% for 2 and 6h p.i. and decreased to 10-12% at 24h, indicating that STy predominantly is localised inside the SCV (Figure 5B).

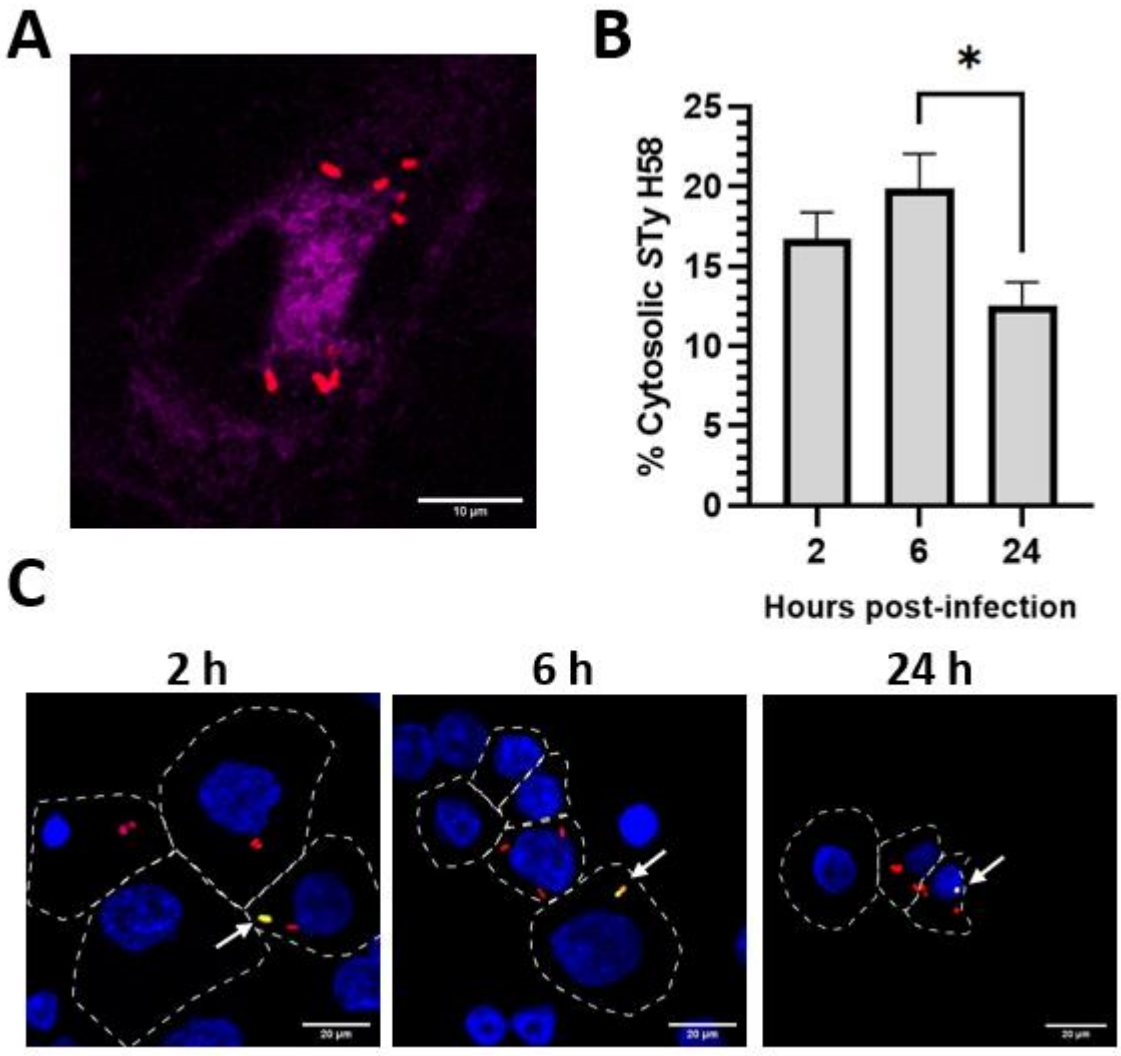

Figure 5. STy is localised to the SCV: THP-1 macrophages were infected with STy expressing mCherry. At specified time points p.i., THP-1 cells were permeabilised with

digitonin. Cytosolic bacteria were labelled using anti-LPS and anti-Vi antibodies, followed by detection using Alexa Fluor 488 secondary antibodies. **A)** Representative confocal image showing no co-localisation of STy and LAMP1 in THP-1 macrophages, Red: vacuolar bacteria, Pink: LAMP1-miRFP703. **B)** Samples were analysed by using ImageXpress Pico Automated Cell Imaging System (see Methods) to obtain the percentage of yellow (cytosolic) labelled bacteria among the red (SCV) population. N = 3, performed in duplicate. Bars: Mean +/- SEM, Statistical analysis by Welch's t-test p-value: *≤ 0.05. **C)** Representative images were taken using a confocal microscope. Blue: DAPI, Red: vacuolar bacteria, Yellow: cytosolic bacteria, Dashed line: THP-1 membrane. Scale bar = 20 µm.

3.2 STy acidifies its cytosol in response to external acid stress:

Our phagosome protection assay experiments demonstrated that STy predominantly localises inside the SCV of macrophages, thereby exposing the bacteria to an acidic environment. As observed with STm, the acidification of the cytosol in response to external acid stress is pivotal for SPI-2 expression and intracellular survival (Chakraborty et al., 2015, 2017; Liew et al., 2019). Our objective was to ascertain whether STy underwent a similar acidification when exposed to acid stress and to elucidate the underlying mechanisms of this adaptation.

Initial experiments were performed using the ratiometric fluorescent probe BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester). BCECF-AM is a well-established tool for detecting intracellular pH in both eukaryotic (Cimprich et al., 1995; Fukushima et al., 1996; Jackson and Halestrap, 1996; Chakraborty et al., 2015) and bacterial systems (Molenaar et al., 1991; Magill et al., 1994; Chakraborty et al., 2015). Both *in vitro* and *in vivo* experiments were conducted to unravel the phenomenon of cytoplasmic acidification in STy. *In vitro* experiments revealed a mean internal pH (pHi) of 6.96 when bacteria were grown at a neutral external pH (pHe) of 7.2. Upon shifting to pHe 5.6, the pHi decreased to 6.60. Parallel observations were made *in vivo* in THP-1 cells, where the inoculum recorded a pHi of 7.05 and observed a reduction in pHi to 6.57 at 2 hours p.i. Interestingly, STy also shows a significant heterogeneity in internal pH both *in vitro* and *in vivo* (Figure

6A). These experiments clearly demonstrate acidification of the STy cytoplasm in response to external acid stress and in response to the acidic SCV environment *in vivo* in THP-1 cells. Although experiments using BCECF-AM provided reliable, gathering *in vivo* data proved difficult due to a loss of signal at later time points.

To address this limitation, we transitioned to using mCherryTYG as a probe for intracellular pH detection. Developed by Dr. Kiran Singh in our lab, mCherryTYG is a M66T mutant of mCherry exhibiting a pH-dependent fluorescence lifetime (Figure 11A, Appendix) (Haynes et al., 2019). Utilising mCherryTYG offers distinct advantages over ratiometric probes such as BCECF. Fluorescence lifetime (FL), being an intrinsic property of the probe, provides a single-channel measurement that is independent of probe concentration, sample absorption, and thickness and is resistant to photobleaching. The pH-dependent FL of mCherryTYG, ranging from pH 9.0 to pH 5.5, demonstrates a consistent reduction of FL.

In vitro experiments were performed to measure the change in pHi in response to changes in pHe. STy expressing mCherryTYG were subjected to acid stress, and FL was measured in neutral pH 7.2 and acidic pH 4.5. The recorded FL was converted to pH values using the standard curve developed for STm (Figure 11B, Appendix). The mean pHi measured decreased from 6.76 to 6.01 when shifted from neutral pHe 7.2 to acidic pHe 4.5 (Figure 6B), Indicating acidification of the STy cytoplasm in response to external acidic pH. These results also validate the ability of mCherryTYG to function as an intracellular pH detection probe. Since most of the STy localises inside the SCV (Figure 6A), the acidic environment would also trigger an acidic reaction by STy. We infected THP-1 macrophages with mCherryTYG-expressing STy and measured the internal pH of individual bacteria inside the macrophages at each time point. Similar to the *in vitro* observations, we saw a sharp drop in the pHi from 6.78 to 6.10 within 2 hours p.i., which gradually lowered to pHi 5.91 at 6 hours p.i. and pHi 5.68 at 24 hours p.i. (Figure 6B). This validates our results with BCECF, that STy acidifies its cytoplasm in response to acidic stress. We also observed heterogeneity in the recorded pHi by mCherryTYG, similar to our observations using BCECF labelling. Interestingly, we observed a decrease in the heterogeneity of the pHi as the infection progressed, indicating a more homogenous population at later time points.

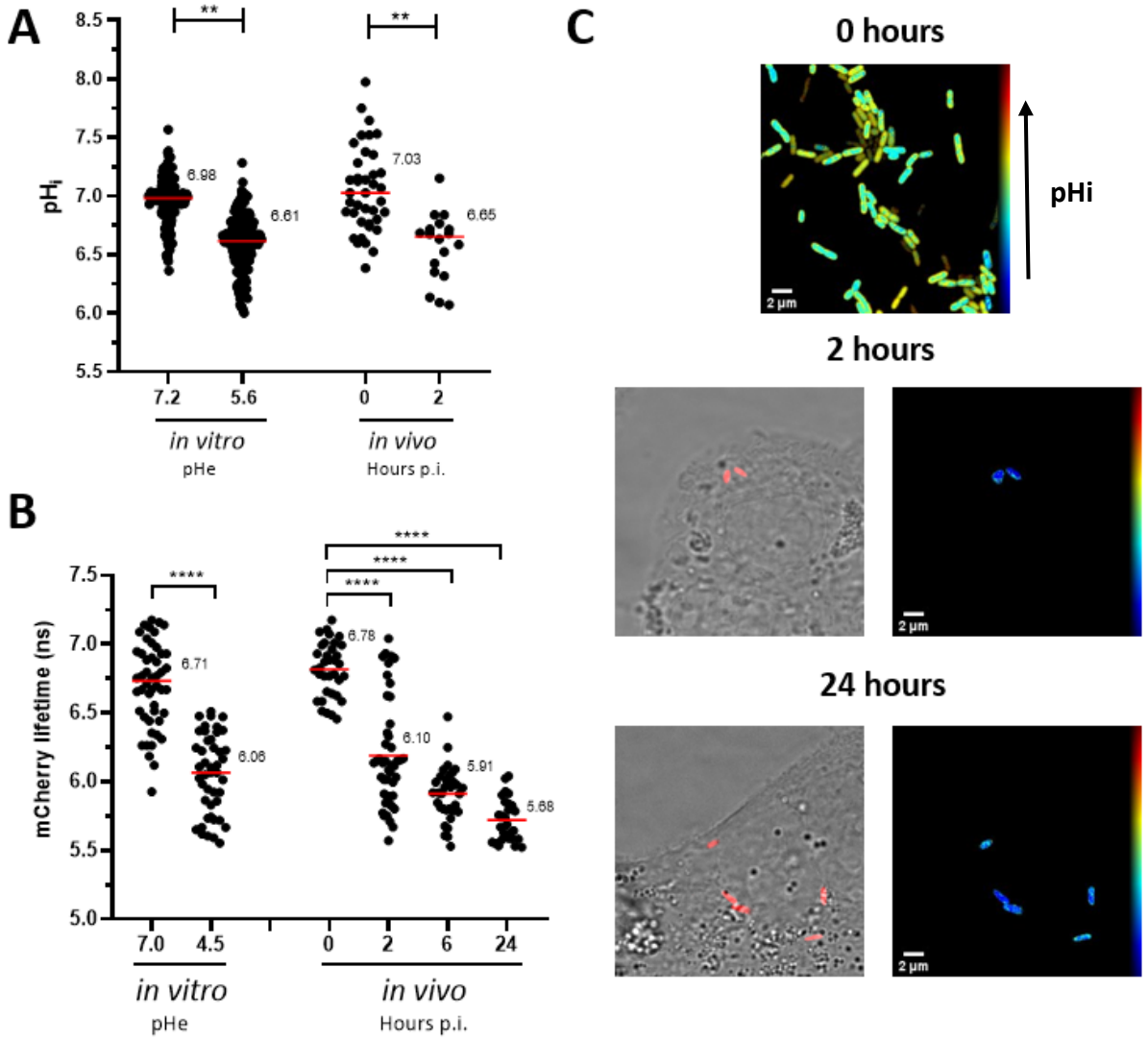

Figure 6. STy acidifies its cytosol in response to an acidic environment: A) STy was incubated with the pH sensitive ratiometric BCECF dye and grown in MGM pH 7.2 before being shifted to MGM pH 5.6 for 1 hour. N=3 (*in vitro*). Bacteria were labelled prior to THP-1 infection (*in vivo*). 0 hours corresponds to the infection inoculum. Using a confocal microscope, emission intensities were recorded at 500-550 nm when excited at 488/440 nm. Ratiometric fluorescence intensity values were converted to pH using a standard curve. One dot represents the pHi recorded in one bacterium. **B, C)** STy expressing pFPV-mcherryTYG were grown *in vitro* in LB pH 7 before and LB pH 4.5 until OD~1 or overnight culture used to infect THP-1 macrophages (*in vivo*). At indicated time points, the mCherryTYG lifetime was recorded by confocal microscopy (see Methods). **B)** One dot represents the lifetime recorded from one bacterium. For all, Bars: Median, Statistical analysis by Welch's t-

test p-value: **≤ 0.01, ****≤ 0.0001. **C)** Representative images of mCherryTYG lifetime recorded for WT STy *in vivo* in THP-1 cells. Colour scale: false colours for recorded internal pH. Scale bar = 2 µm

3.4 SsrB is required to maintain STy infection in THP-1 macrophages:

Unlike in STm, the role of SPI-2 in STy survival in macrophages is not well characterised (see Introduction). Previous investigations have provided contrasting conclusions (Forest et al., 2010; Reuter et al., 2021; Hamblin et al., 2023). Moreover, these studies employed diverse techniques and investigated the role of specific SPI-2 genes rather than providing a mechanistic understanding, leaving this question unanswered. We decided to re-investigate the role of SsrB, which has been characterised as the master regulator of SPI-2 genes in STm and is essential for intracellular survival in response to an acidic cytosol (Feng et al., 2003; Walthers et al., 2011; Liew et al., 2019).

Initially, we utilized the gentamicin protection assay to assess intracellular bacterial replication dynamics. THP-1 macrophages were infected with both the *ssrB* null mutant and WT STy strains, followed by cell lysis at 30 minutes, 2 hours, 6 hours, and 24 hours p.i. to release intracellular bacteria. Enumeration of bacterial colonies on LB agar plates allowed for CFU quantification. Consistent with the existing literature, no significant difference in CFU count was noted between the *ssrB* null mutant and wild-type strains (Figure 7A). However, drawing a definitive conclusion from these results regarding the role of *ssrB*, and by extension SPI-2, in intracellular STy survival in macrophages would be premature. Ensemble-based assays such as the gentamicin protection assay have limitations, as they overlook host cell death induced by the pathogen, potentially leading to an underestimation of the bacterial load. As elucidated by Hamblin *et al.*, 2023, alternative assays such as the fluorescence dilution assay may provide deeper insights into replication rates obscured by CFU measurements.

Most other techniques fail to characterise intracellular replication rates on a single cell basis as they quantify the population level phenotypes. Therefore, we opted to use TIMER^{bac} as a reporter for monitoring intracellular replication rates of STy. TIMER^{bac}, being genetically encoded, is not affected by dilution due to cell division. It

facilitates monitoring of replication states exhibited by individual bacteria rather than as an ensemble of the entire population. By its nature of reversibility, TIMER^{bac} can detect any re-initiation of bacterial replicative phase without any dependency on its original state, and thus allow the tracking of replication kinetics throughout infection. TIMER^{bac} exhibits a shift in its emission spectrum from green to red as it matures, providing a reflection of the bacterial replication rate. Highly replicative bacteria constitutively express TIMER^{bac}, resulting in a higher green/red ratio. In contrast, slow-replicating bacteria accumulate higher levels of matured TIMER^{bac} protein without producing new green ones, leading to a lower green/red ratio (Figure 7B). TIMER^{bac} has been previously employed to characterise various replication states of STm in epithelial cells and mouse models (Claudi *et al.*, 2014; Luk *et al.*, 2021).

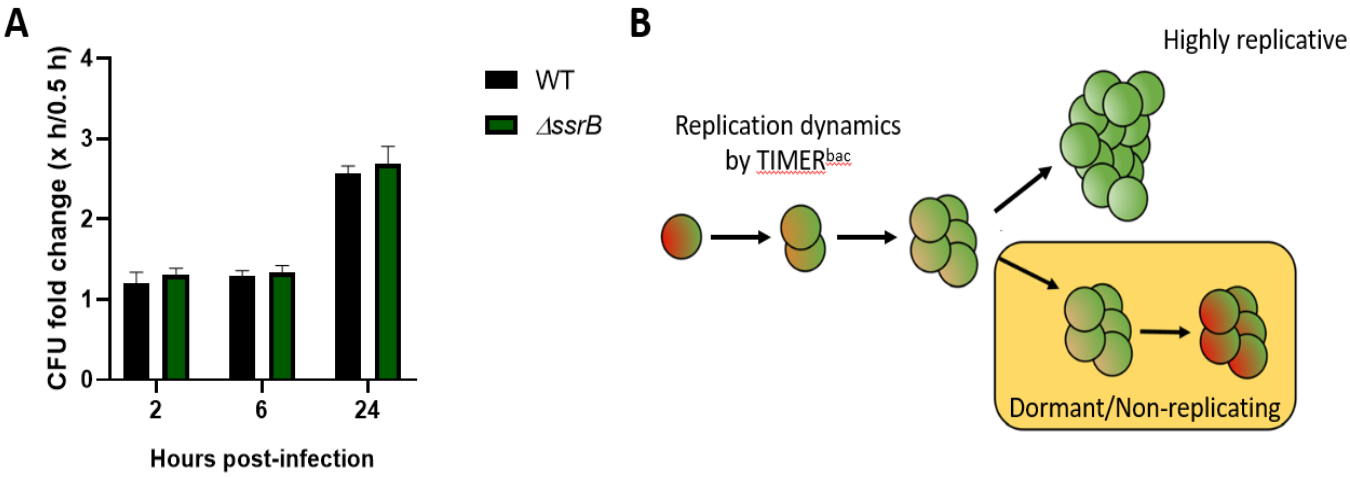

Figure 7. CFU count is not a reliable measure of intracellular bacteria replication rate: A) THP-1 macrophages were infected with WT and *ssrB* null STy strains. The bacteria burden was estimated for each time point by CFU count (see methods, Gentamicin protection assay). Plotted as fold change of uptake (CFU count at x hours p.i. / CFU count at 30 mins p.i.). Bars: Mean +/- SEM. **B)** Illustration showing the use of TIMER^{bac} for accessing intracellular bacterial replication rates. At a high replication rate, the production of new green TIMER proteins outpaces the maturation to the red state, while in non-replicative states, the existing TIMER proteins matures to the red state with no new green TIMER being produced (yellow box).

THP-1 macrophages were infected with STy strains expressing *TIMER*^{bac}. Intracellular bacteria were extracted at specified time points by lysing THP-1 macrophages, followed by flow cytometry analysis. Fluorescence intensities for the green (Ex488/Em510) and red (Ex562/Em580) states of the protein were recorded for each bacterium, and frequency distributions of green/red ratios were plotted for comparison (Figure 9A). As the infection progressed, we observed a gradual lowering of replication rates for both WT and *ssrB* null mutant strains. At 30 min, 2 and 6 hours p.i., both the WT and the *ssrB* null mutant exhibited similar replication rates. However, at 24 hours p.i., a significant divergence was observed between WT and *ssrB* null mutant strains, with WT showing a lower replication rate compared to its *ssrB* null counterpart (compare blue solid and dotted lines, Figure 8A). Furthermore, the WT displayed a biphasic distribution (black and blue solid lines, Figure 8A), indicating the presence of two distinct intracellular populations with varying replication rates, which was absent in the *ssrB* mutant (black dotted line, Figure 8A). Supporting these observations from flow cytometry analysis, we also observed similar trends via confocal microscopy imaging (Figure 8B).

Our TIMER^{bac} experiments revealed a heightened replication rate in the *ssrB* null background, suggesting a potential increase in host cell death due to the elevated bacterial load. Encouraged by these findings, we aimed to investigate the cytotoxic effects exerted by both WT STy and its *ssrB* null mutant in THP-1 macrophages. To accomplish this, we employed the CytoTox 96 kit from Promega. We anticipated that this assay would not only shed light on the cytotoxicity induced by the bacterial strains, but also serve as a means to validate the limitations associated with CFU counting as a measure of bacterial load.

THP-1 macrophages were infected with both the *ssrB* null mutant and WT STy strains, and supernatant was collected at 30 min, 2 hours, 6 hours, and 24 hours post-infection (p.i.) for the cytotoxicity assay. Following supernatant collection, THP-1 cells were lysed and released bacteria plated on LA plates for CFU quantification. Host cell cytotoxicity was calculated for each strain at each time point following the kit protocol and data was plotted for comparison (Figure 9A). As expected, we observed a gradual increase in THP-1 cytotoxicity for both strains as the infection progressed. At 30 min, 2 hours, and 6 hours p.i., both the WT and *ssrB* null mutant exhibited similar cytotoxic effects on THP-1 cells. However, at 24 hours p.i., we observed an elevation in THP-1

cytotoxicity induced by the *ssrB* null mutant strain compared to the WT. Intriguingly, there was no discernible difference in CFU counts between the WT and *ssrB* null mutant strains (Figure 9B). These findings highlight the limitations of CFU counting for quantifying intracellular bacteria. Despite similar CFU counts, the *ssrB* null mutant displayed a higher replication rate, leading to increased host cell death as revealed by the cytotoxicity assay. Moreover, these observations suggest an alternative role played by SsrB in maintenance of STy infection within macrophages.

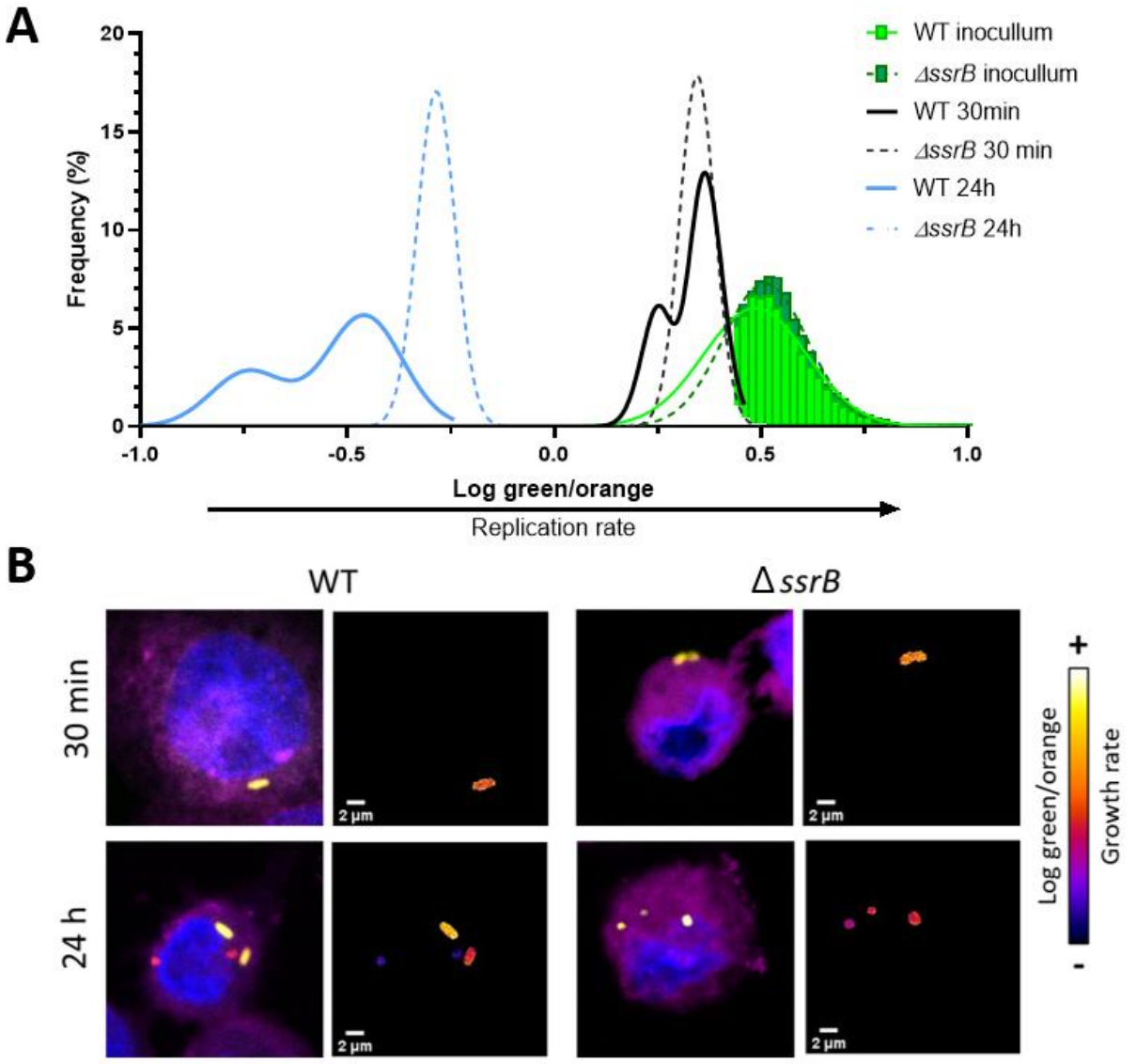

Figure 8. The *ssrB* null strain exhibits a less heterogeneous and higher growth rate *in vivo*: **A, B)** *In vivo* replication rates were measured using TIMER^{bac}. THP-1 macrophages were infected with WT and *ssrB* null STy strains expressing TIMER^{bac}. **A)** At the indicated time points, p.i., bacteria were extracted by lysing THP-1 macrophages and analysed by flow cytometry. n>2000 per strain, per time point per replicate. N=2, **B)** In parallel, infected THP-1 macrophages were fixed with PFA and imaged under a confocal microscope. For both sets of samples, fluorescence intensities from green (Ex:488 nm, Em:510 nm) and red (Ex:562 nm, Em:580 nm)

channels were recorded for each bacterium and green/red ratios were plotted. Colour scale: bottom right. Scale bar = 2 µm

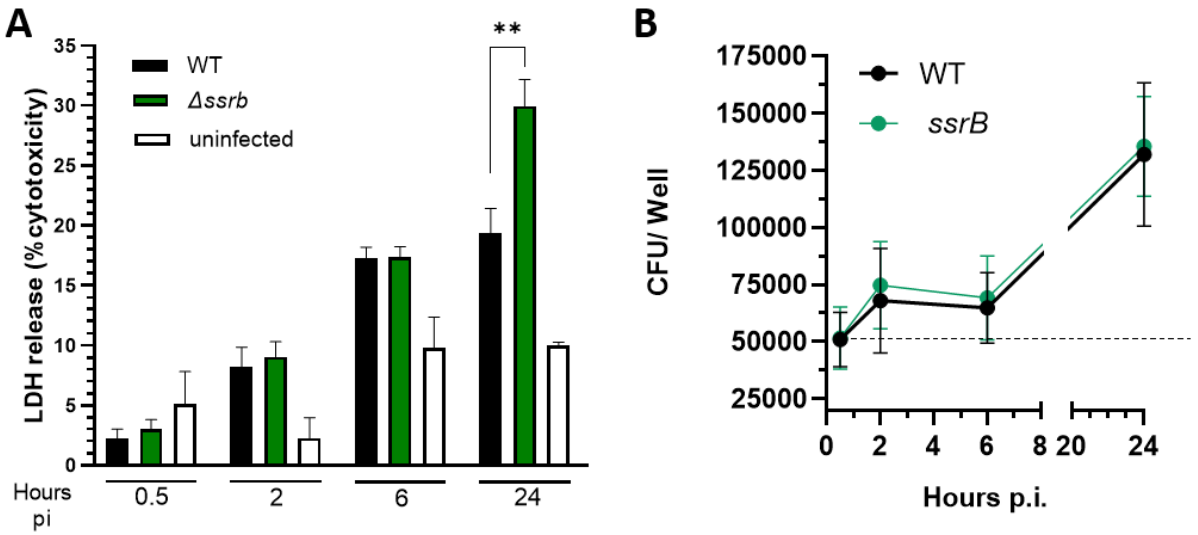

Figure 9. The *ssrB* null strain exhibits higher cytotoxicity in THP-1 macrophages: A, B) THP-1 macrophages were infected with WT and *ssrB* null STy strains. **A)** At the indicated time points p.i., supernatants from infected wells were collected and subjected to a cytotoxicity assay. The percentage of THP-1 cell death was calculated at each time point. **B)** A gentamicin protection assay was performed on the same wells, and CFU numbers were quantified on LA plates. Bars: Mean +/- SEM. n=4 per strain, per time point. N=3.

3.5 *sifA* expression is SsrB-dependent:

Previous studies conducted in our laboratory have elucidated the role of SsrB in the regulation of SPI-2 genes in STm. Specifically, Walthers et al. demonstrated the direct binding of SsrB to promoter sequences of *sifA, sifB,* and *sseJ* in STm (Walthers *et al.*, 2011). Furthermore, studies have shown that SsrB exhibits enhanced binding capacity to DNA under acidic conditions, thereby linking cytoplasmic acidification to SPI-2 gene expression in STm (Liew *et al.*, 2019; Shetty and Kenney, 2023). In order to understand SPI-2 gene activation in STy, we decided to examine the *sifA* promoter activity in WT and *ssrB* null environments under neutral and acidic conditions. *sifA* was selected, as it has been shown to be essential for the maintenance of the SCV membrane and vacuole trafficking, and is essential for STm survival in multiple host cell models (Beuzón *et al.*, 2000, 2002). A recent publication has also demonstrated that the STy *sifA* mutant is defective for replication in THP-1 macrophages (Hamblin *et al.*, 2023).

For this analysis, we developed a dual fluorescent protein (FP) reporter plasmid. This plasmid featured a constitutive expression of mCherry under the *tet* promoter and a fusion of the promoter of *sifA* with *tagBFP* (pZS mCherry P*sifA*-tagBFP). The constitutive expression of mCherry served as a normalisation control, mitigating variations in the plasmid copy number carried by individual bacteria. We anticipated a substantial reduction in the activation of *PsifA::tagBFP* in the *ssrB* null background if the regulatory mechanism observed in STm also holds true for STy.

For *in vitro* studies, WT and *ssrB* null mutant strains carrying the pZS mCherry P*sifA*-tagBFP were grown in LB at neutral pHe 7.0 and SPI-2-inducing acidic pHe 4.5 and fluorescence intensities for mCherry and tagBFP were recorded using confocal microscopy. The ratios of tagBFP/mCherry were plotted (Figure 10A). The WT strain exhibited an increase in *PsifA* activity at pHe 4.5 compared to pHe 7.0, indicating SsrB activation of the *sifA* promoter. At the same time, no increase in *sifA* promoter activity was observed in the *ssrB* mutant strain under acidic conditions. Interestingly, the WT strain showed a significant increase in heterogeneity in *PsifA* activity at acidic pHe, with only a slight increase in heterogeneity observed in the *ssrB* mutant. Notably, both the WT and *ssrB* mutant strains displayed non-zero *PsifA* activity at neutral pHe,

suggesting a basal level of activation of the *sifA* promoter even under non-inducing conditions.

To further validate the role of cytoplasmic acidification in the activation of *sifA* expression, *PsifA* activity was assessed under non-acidifying conditions. *PsifA* activity was examined in the WT strain during over-expression of *cadC/BA* at both neutral and acidic pHe. Over-expression of *cadC/BA* prevents cytoplasmic acidification of bacteria, thus negating the expected increase in *PsifA* activation under acidic pHe. As hypothesised, we observed no elevation in *PsifA* activity at acidic pH; instead, a decrease was observed compared to neutral pHe (Figure 10A). This finding validates that STy exhibits SsrB-dependent activation of *sifA* expression under SPI-2 inducing conditions.

To analyse *PsifA* activation *in vivo*, THP-1 cells were infected with WT and *ssrB* null mutant strains carrying the dual FP plasmid. Intracellular bacteria were isolated at specified time points by lysing THP-1 cells, followed by flow cytometry analysis. Fluorescence intensities were recorded for mCherry and BFP from each bacterium. The threshold intensity for BFP-positive bacteria was defined by using a BFP null strain (WT pZS mcherry). Frequency distributions of tagBFP/mCherry intensity ratios were plotted for comparison and the percentage bacteria showing P*sifA* activity from the total population was calculated at each indicated time point (Figure 10B). At 30 mins, 2 and 6 hours p.i. *PsifA* activity of both the WT and *ssrB* mutant was absent. However, at 24 hours p.i., the WT strain exhibited an increase in the number of *PsifA* positive bacteria from 3.5% to 33%, while the *ssrB* null mutant showed no increase. This is consistent with our *in vitro* data, indicating that *sifA* expression in STy is SsrB-dependent *in vivo* in THP-1 cells, and its expression occurs between 6 and 24 hours p.i.

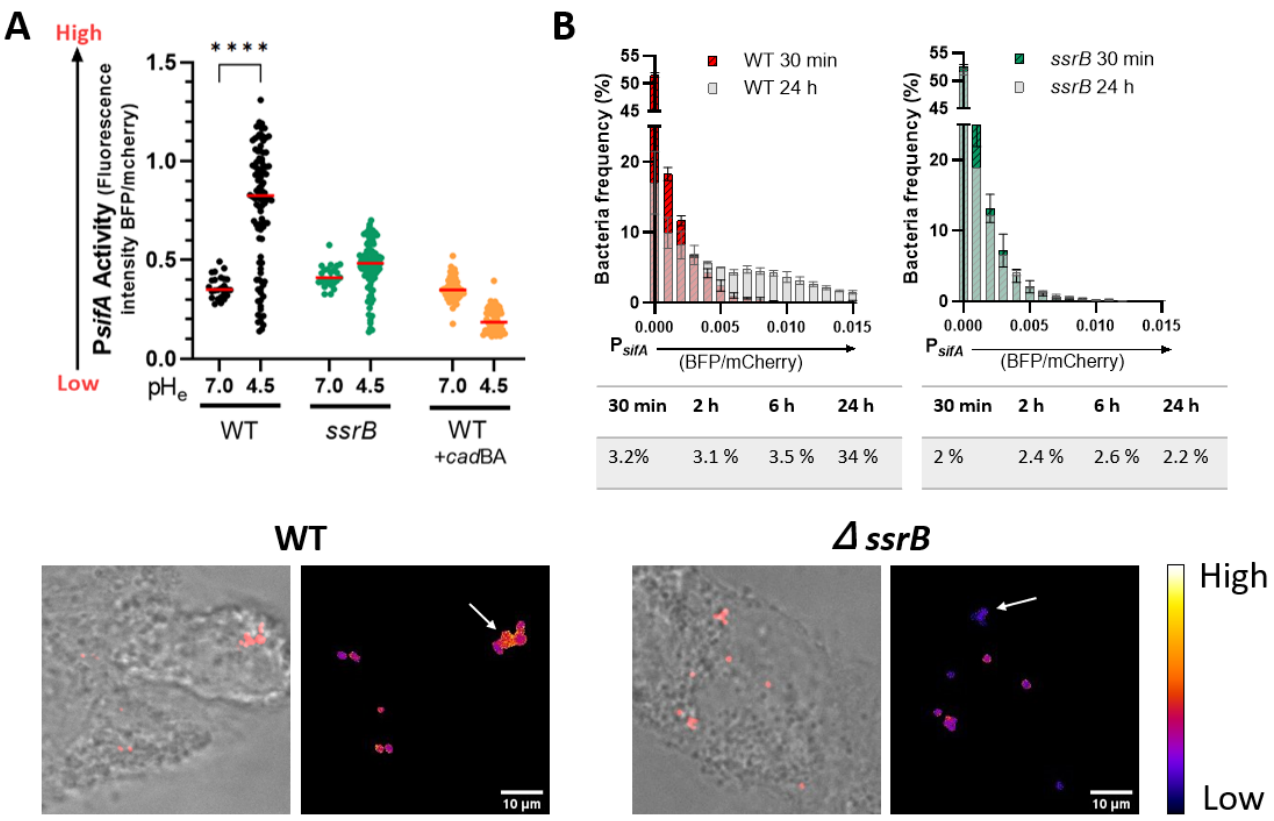

Figure 10. *sifA* expression in STy is SsrB-dependent: A, **B)** *PsifA* transcriptional activity was measured for WT and the *ssrB* null mutant using pZS mCherry PsifA-tagBFP. **A)** Bacteria were grown *in vitro* under neutral or acidic conditions prior to analyses. Each dot represents the BFP/mCherry ratio recorded from a single bacterium. n>50 per replicate, N=2, p<0.0001. Bars: median, Statistical analysis by Welch's t-test p-value: ****≤ 0.0001. **B)** THP-1 macrophages were infected with STy strains carrying the reported plasmid. At each time point, p.i. bacteria were extracted by lysing THP-1 macrophages and analysed by flow cytometry. In parallel, samples were fixed with PFA and imaged using a confocal microscope. BFP to mCherry fluorescence intensity ratios were calculated for each bacterium. Bottom panel: frequency distribution for BFP/mCherry ratios are presented. n>5000 per strain per replicate per timepoint from 2 independent experiments. Error bars: SEM. Percentage of BFP positive bacteria indicated for each time point p.i., Bottom: representative confocal images at 24 h p.i. Right: Colour bar indicating *PsifA* activity. Scale Bar: 10µm.

4. Discussion

Many studies have elucidated the pathways of *Salmonella enterica* pathogenesis using the serovar Typhimurium as model. While systemic murine infection with STm closely mimics systemic disease in humans, the genetic disparities between STm and STy hinder the direct translation of findings into an understanding of human-specific virulence mechanisms. To bridge this gap, we used the human-derived macrophage cell line THP-1 to study STy pathogenesis to understand the underlying mechanisms of infection. Furthermore, we used STy strain H58, a clinically prevalent typhoidal strain in Africa and South east Asia (Feasey *et al.*, 2015; Pham Thanh *et al.*, 2016; Yan *et al.*, 2016).

4.1 STy regulatory pathways conforms to the STm model:

STy is mainly localised in the SCV:

Bacterial localisation is a crucial determinant of intracellular lifestyle, with distinct niches such as the SCV and the host cytosol providing vastly different environments for bacterial growth and survival. Initially, we sought to quantify the vacuolar populations by labelling the LAMP1 endosomal protein. However, our fixed-cell immunostaining of LAMP1 protein yielded inconsistent results. echoing previous findings indicating the loss of membrane integrity in fixed samples (Rajashekar et al., 2014). To overcome this challenge, we developed a THP-1 cell line expressing the LAMP1-miRFP703 fusion protein. Surprisingly, similar to fixed samples, there was no consistent co-localisation of LAMP1 with STy (Figure 5A), raising questions as to potential differences in SCV maturation events during STy infection of macrophages compared to STm.

Nevertheless, to ascertain the distribution of STy localization, we employed a phagosome protection assay. Our findings revealed that STy is predominantly localized within the SCV, with a minor population detected in the cytoplasm, which diminishes at later stages of infection (Figure 5B).

STy acidifies its cytoplasm in macrophages:

Localisation of STy in the SCV exposes the pathogen to an extremely acidic environment reaching as low as pH 5 (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996). To investigate the response of STy to external acidic stress, we utilised an in-house-developed pH detection probe, mCherryTYG. Our findings reveal that STy acidifies its cytoplasm significantly to pH 6.10 as early as 2 hours p.i., followed by a further reduction to pH 5.68 at 24 hours p.i. (Figure 6B). Interestingly, we observed a significant heterogeneity in internal pH at each time point. These results were validated by our similar observations using the ratiometric probe BCECF-AM pH (Figure 6A).

Overall, our findings shed light on the dynamic interplay between STy and its acidic intracellular environment, highlighting the ability of the pathogen to modulate its internal pH in response to external acidic stress. Further, we plan to investigate the role of pH-sensitive two-component systems such as OmpR/EnvZ and PhoQ/P, which have been identified by our lab as key drivers of the acid stress response in STm.

***sifA* expression is SsrB-dependent:**

Previous studies from our lab have demonstrated the role of cytoplasmic acidification for expression of *ssrB*, which then activates expression of SPI-2 genes (Chakraborty *et al.*, 2015, 2017; Kenney, 2019; Liew *et al.*, 2019). The expression of SPI-2 genes is essential for intracellular survival of STm (Ochman *et al.*, 1996; Hensel *et al.*, 1998; Beuzón *et al.*, 2002). While studies have assumed that STy follows the SPI-2 regulatory pathway similar to STm, this assumption has not been validated in STy.

To understand the role of SsrB in regulating SPI-2 genes in STy, we investigated *sifA* expression. Studies in STm have characterised the role of SifA in maintaining SCV membrane integrity during infection. Recent studies of STy have also shown a replicative defect *sifA* mutant in macrophages (Hamblin *et al.*, 2023). In line with established models, our *in vitro* experiments revealed that the WT strain activated the *sifA* promoter in an acidic environment. However, this activation profile was not observed in either *ssrB* null and *cadC/BA* over-expressed backgrounds, validating the acid-dependent activation of *sifA* in STy by SsrB (Figure 10A). Notably, we observed a basal level of *sifA* promoter activity in all conditions, suggesting potential leaky expression of *sifA* in STy. *In vivo* experiments in THP-1 cells further supported our

findings, with the WT strain exhibiting an increased number of bacteria with activated *sifA* promoter at 24 hours p.i., a response that was absent in the *ssrB* null strains (Figure 10B). These observations underscore the acid-dependent role of SsrB in *sifA* expression. Conducting similar assays with other SPI-2 genes could provide deeper insights into the STy SsrB regulon.

4.2 SsrB is required for persistent infection in STy:

While the significance of SPI-2 in *Salmonella* Typhimurium (STm) infection is well-documented, the understanding of its role in *Salmonella* Typhi (STy) pathogenesis remains less clear. Previous studies have generated conflicting findings regarding the involvement of SPI-2 in STy infection. Here, we investigated the role of SPI-2 for STy pathogenesis in WT and *ssrB* null backgrounds.

Consistent with published findings using the gentamicin protection assay, we observed no replication defect in the *ssrB* null mutant strain compared to the WT strain. However, our results from TIMER^{bac} identified a role for SsrB during infection. At 30 mins, 2 and 6 hours, there was no difference in replication rates between the WT and *ssrB* null strain, but at 24 hours p.i. the *ssrB* null strain showed a higher replication rate compared to the WT strain (Figure 8A). We hypothesized that the difference in reported phenotype between the assays could be due to the underestimation of CFUs by the gentamicin protection assay due to host cell death.

To further validate this hypothesis, we examined host cell cytotoxicity in THP-1 cells by WT and *ssrB* null mutant STy strains. Consistent with our TIMER^{bac} results, we observed a higher host cell death in the null mutant strain compared to WT, but only at 24 hours p.i. (Figure 9A). In contrast, there was no difference in CFUs from our gentamicin protection assay performed in parallel (Figure 9B). These results validate the short comings of the gentamicin protection assay for quantification of intracellular bacterial replication. These results also point to a previously unreported role for SsrB in maintaining a persistent macrophage infection by STy.

A review of these observations revealed that similar observations have been reported for STm infection in mice phagocytes for *ssaV,* a SPI-2 gene that is essential for assembly of the T3SS2. Despite reduced growth, the *ssaV* mutant exhibited a

higher bacterial load per host cell compared to the WT strain (Grant *et al.*, 2012). Additionally, studies of host cell cytotoxicity have revealed that SPI-2 activation in *Salmonella* Typhimurium delays host cell death, while high SPI-1 activity triggers caspase-1-mediated apoptosis (Monack *et al.*, 2001; Knodler *et al.*, 2010). Hamblin *et al.* have shown that the T3SS1 can partially compensate for the functions of T3SS2 by translocating SPI-2 effectors. These observations along with prior literature gives rise to multiple possible explanations. It is conceivable that the loss of *ssrB* results in heightened T3SS1 activity to make up for the absence of T3SS2 function. This increased SPI-1 activation may contribute to elevated host cell death. A complete identification of the genes regulated by SsrB during infection might help to understand the additional pathways regulated by SsrB apart from its role in pathogenesis. Further investigations into the secretion of various apoptotic factors by the host cell could provide valuable insights into deciphering this observation.

Exploring the metabolic cost associated with the expression of T3SS and effector translocation represents an intriguing research avenue. Single cell analysis of STm have shown that only 17% of bacteria expressed the T3SS2 under *in vitro* inducing conditions (Liew *et al.*, 2019). Studies have also demonstrated that the expression of T3SS entails a significant metabolic cost and can even result in retarded growth *in vitro* (Sturm *et al.*, 2011; Jennings *et al.*, 2017). These observations open up an interesting question for *Salmonella* pathogenesis. Further analysis of *in vivo* T3SS2 expression levels and single cell analysis on effector translocation by STy would allow us to gather more insights into the metabolic cost of T3SS2 expression and understand the trade-offs made by the pathogen for persistent infection.

In summary, our study sheds light on the pathogenesis of STy, revealing both similarities and novel mechanisms compared to the model developed using STm. We demonstrate that, like STm, STy maintains its localisation to the SCV and responds to the acidic environment by acidifying its own cytosol. Additionally, we validate the acid-dependent expression of *sifA* by SsrB, both *in vitro* and *in vivo*. However, our investigation into the role of SsrB in STy pathogenesis unveils a novel finding. We find that the absence of SsrB leads to a significantly higher intracellular replication rate of STy compared to the wild-type strain, accompanied by increased host cell death.

These results suggest that SsrB is crucial for maintaining persistent STy infection in macrophages. Moving forward, further analysis is needed to unravel the underlying mechanisms driving this observation.

5. Appendix:

Live tracking of intracellular pH of *Salmonella* during infection:

As described in the Introduction, pH sensing is an essential phenomenon for the regulation of virulence factors in *Salmonella*. The stress response regulators, SsrA/B, EnvZ/OmpR, and PhoQ/P are all pH-sensitive and are essential for the activation of SPI-2 gene expression (Chakraborty et al., 2015, 2017; Liew et al., 2019; Shetty and Kenney, 2023). This sensitivity underscores the importance of monitoring intracellular pH changes, especially given the rapid acidification of the SCV. By tracking these pH fluctuations, we can gain invaluable insights into the sequential responses and strategies employed by *Salmonella* within the host.

While a variety of pH detecting probes are available, they come with their own set of limitations. Among them, BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) stands out as one of the most commonly used pH probes. BCECF-AM is a cell-permeant dual excitation dye that exhibits pH-dependent ratiometric emissions. It has been extensively employed for detecting intracellular pH in both eukaryotic (Cimprich et al., 1995; Fukushima et al., 1996; Jackson and Halestrap, 1996) and bacterial systems (Molenaar et al., 1991; Magill et al., 1994; Chakraborty et al., 2015). However, despite its widespread use, BCECF-AM presents certain limitations, including uneven loading of the dye, leakage from cells, and dilution of signal intensity loss due to cell division.

Previously, our lab has pioneered the use of a FRET-based pH sensor known as the I-switch for detecting intracellular pH in STm (Chakraborty et al., 2015). The I-switch is DNA biosensor, which has several advantages over other probes. It was efficiently taken up by *Salmonella* and did not exhibit any leakage from the cell, even retaining the probe after spheroplast preparation from the transformed cells. Additionally, the I-switch demonstrates reversible behaviour, allowing it to track acid stress recovery in STm. Although the I-switch proved effective for extended periods due to the slowed replication rate *in vivo*, it poses limitations from probe dilution by cell division. Previous investigations conducted in our laboratory have highlighted the inefficacy of other ratiometric probes, such as pHluorin, in detecting intracellular pH in

Salmonella. Unlike other probes, pHluorin exhibited notable levels of heterogeneity, which were not observed using BCECF or the I-switch, and it produced weak signals even under constitutive expression conditions (Morimoto et al., 2011; Chakraborty et al., 2017).

To address these limitations, Dr. Kiran Singh from our laboratory recently developed mCherryTYG, a pH-sensitive variant of mCherry (M66T) that exhibits pH-dependent fluorescence lifetime (FL). Utilising mCherryTYG offers distinct advantages over ratiometric probes such as BCECF and pHluroin. The fluorescence lifetime is an intrinsic property of the probe, provides a single-channel measurement independent of probe concentration, sample absorption, and thickness and is resistant to photobleaching. Moreover, since it is a genetically encoded probe, it allows for prolonged tracking of intracellular pH without any dilution due to cell division. mCherryTYG demonstrates a pH detection range from pH 9.0 to pH 5.5 and exhibits a consistent reduction in FL corresponding to a lowering of environmental pH. mCherryTYG is currently being characterised in STm in multiple host models, including: HeLa cells and heterologous hosts C. elegans and Danio rerio by Dr. Kiran Singh along with Dr. Marion Fernandez and Dr. Rahul Dilawari. This comprehensive characterization aims to elucidate the utility of mCherryTYG for monitoring intracellular pH dynamics in the context of diverse *Salmonella* lifestyles.

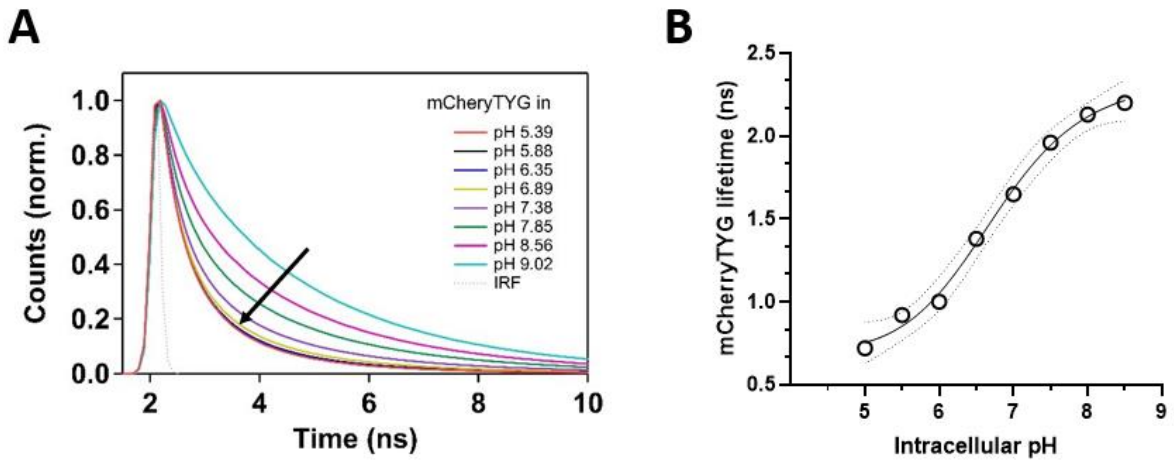

Figure 11. mCherryTYG shows pH dependent fluorescence lifetime: A) Fluorescence decay curves recorded by time-resolved measurements of purified protein show a decrease in fluorescence lifetime with decreasing pH. **B)** The

fluorescence lifetime to pH conversion standard curve was recorded for STm clamped to indicate pH values. (Reproduced using data from Dr. Kiran Singh, unpublished).

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