THE UNIVERSITY
of EDINBURGH

Investigating the molecular mechanisms underlying the differential virulence of *Salmonella enterica* serovars Typhimurium and Choleraesuis

Imogen Rena Johnston-Menzies

A thesis submitted for the degree of Doctor of Philosophy (PhD)

College of Medicine and Veterinary Medicine

The University of Edinburgh

2021

Declaration

I declare that this this thesis was written and composed by me and the work presented is entirely my own unless otherwise specified. This work has not been submitted for any degree or professional qualification.

Imogen Johnston-Menzies, 2021

Abstract

Salmonella enterica is a facultative intracellular pathogen which impacts human and animal health globally. Livestock animals, as a source of nutrition and environmental contamination, represent a significant reservoir for human non-typhoidal salmonellosis. For *Salmonella enterica* serovars, systemic dissemination to sites outwith the intestinal environment is a phenotypic marker of bacterial host-adaptation, a trait which influences infection outcome. Host-adapted *Salmonella* Choleraesuis, for example, disseminates to the bloodstream, liver, and spleen of infected pigs, causing little intestinal inflammation but greater mortality whilst host-generalist *Salmonella* Typhimurium causes self-limiting enteritis.

S. enterica encodes two type III secretion systems (T3SSs) which have critical roles in bacterial pathogenesis – the T3SS-1 and T3SS-2. Both systems are molecular syringes that inject effector proteins into host cells. These effectors manipulate host-cell processes to aid either invasion (T3SS-1) or intracellular survival (T3SS-2). As significant evidence suggests that type III secretion influences host adaptation, the first aim of this study was to characterise and compare the secretome of host-generalist *S.* Typhimurium and host-adapted serovar *S.* Choleraesuis under T3SS-1 inducing conditions.

A label-free quantitative proteomics approach was used to characterise the secretome of two strains of well-defined virulence in livestock animals – *S.* Typhimurium ST4/74 and *S.* Choleraesuis SCSA50 – and identified key differences between the strains. This was the first comprehensive and quantitative comparison between the secretomes of two different non-typhoidal *S. enterica* serovars and demonstrated that ST4/74 secreted more T3SS-1 effectors than host-adapted SCSA50, a result which could not have been predicted from the genome sequences alone. In addition, the proteomic approach also identified several hypothetical proteins which were investigated for T3SS-dependent secretion.

The subsequent aim of this study was to unravel regulatory differences between ST4/74 and SCSA50 at the transcriptional level using quantitative reverse transcription PCR (RT-qPCR). Initial comparative bioinformatic analysis revealed single nucleotide polymorphisms in important regions of the promoters of several factors which control T3SS-1 expression including major transcriptional regulators HilA and HilE. Consequent RT-qPCR investigation uncovered small but biologically relevant increases in transcriptional expression of T3SS-1 transcriptional activators by ST4/74 compared to SCSA50 which could explain the differences in effector protein secretion.

The importance of the T3SS is, however, in the context of host cells. The last aim of this study was therefore to examine the interactions of ST4/74 and SCSA50 with porcine cell lines using assays to assess invasion, intracellular survival, net replication, and intracellular cytosolic populations. Whilst the strains were similarly invasive, their intracellular lifestyles differed – in the intestinal cell line IPEC-J2, ST4/74 replicated faster and had a larger cytosolic population than SCSA50, two bacterial intracellular strategies linked to activity of T3SS-1.

Overall, this study revealed significant differences in the repertoire and amount of secreted proteins by host-generalist ST4/74 and host-adapted SCSA50. Although more mechanistic exploration is required, increased transcriptional expression of T3SS-1 transcriptional activators by ST4/74 potentially influences this. Paired with the differences in intracellular lifestyle, this investigation identified type III secretion as a major differentiating factor between *S.* Typhimurium and *S.* Choleraesuis and this data could inform on new strategies for crucial broad-spectrum vaccine design.

Lay Summary

Salmonella enterica (*S. enterica*) is an infamous bacterial pathogen which causes food poisoning in humans, greatly impacting human and animal health globally. Livestock animals, as a source of nutrition and environmental contamination, represent a significant reservoir for human salmonellosis. There are over 2,600 types of *S. enterica* called serovars, which are defined by the proteins decorating the bacterial surface and by the severity of disease they cause. For *S. enterica* serovars, dissemination to sites outwith the gut is a marker of *Salmonella* host-adaptation, a trait which influences the outcome of infection. Host-adapted *Salmonella* serovar Choleraesuis, for example, spreads to the bloodstream, liver, and spleen of infected pigs, causing little gastrointestinal distress but greater mortality. In contrast, host-generalist *Salmonella* Typhimurium causes classical food poisoning symptoms. Little is known about why these serovars behave so differently.

To invade and survive within mammalian cells, *S. enterica* uses two type III secretion systems (T3SSs). T3SSs are molecular syringes that inject toxins called effector proteins from the bacteria into mammalian host cells to manipulate host cell processes to aid invasion and to suppress the immune response. As significant evidence suggests that these effector proteins influence host-adaptation, the first aim of this study was to compare the secretion of effectors by host-generalist *S.* Typhimurium and host-adapted *S.* Choleraesuis.

A biochemical technique called proteomics was used to identify and quantify the amount of effector proteins secreted by *S.* Typhimurium and *S.* Choleraesuis under laboratory conditions. This was the first comprehensive and quantitative comparison of protein secretion between two different livestock-associated *S. enterica* serovars and demonstrated that *S.* Typhimurium secreted more effector proteins than *S.* Choleraesuis.

The subsequent study aim was to unravel regulatory differences between the two serovars by measuring how active the positive and negative regulators that control the T3SSs were. Small but biologically relevant increases in positive regulators of T3SS in *S.* Typhimurium compared to *S.* Choleraesuis were uncovered, which could explain the differences in effector protein secretion.

To *Salmonella*, the greatest importance of the T3SSs is in contact with mammalian cells. The last aim of this study was therefore to examine the interactions between *S.* Typhimurium and *S.* Choleraesuis with immortal cell lines to assess bacterial invasion, survival and replication. Whilst the serovars were similarly invasive, it was discovered that *S.* Typhimurium replicated faster than *S.* Choleraesuis – a bacterial strategy linked to activity of the T3SSs.

Overall, this study revealed significant differences in the repertoire and amount of secreted effector protein toxins by host-generalist *S.* Typhimurium and host-adapted *S.* Choleraesuis. Although more exploration is required, increased activity of positive regulators of the T3SSs potentially influences this. Paired with the differences in bacterial interactions with host cells, this investigation identified type III secretion as a major differentiating factor between the two serovars and the results could inform on new strategies for crucial broad-spectrum vaccine design.

Acknowledgements

The first person I would like to thank is my supervisor Jo Stevens without whom I do not think finishing my PhD would have been possible. I want to thank her for being the most approachable, patient and kind mentor who has taught me how to be a good scientist and truly what kind of leader I would like to be. I also want to thank my secondary supervisor Prerna Vohra for helping me challenge new ideas, being a *Salmonella* cheerleader and for being an early career inspiration to me.

I cannot thank all of the members of the MMBP lab group enough for their encouragement and help in the lab over the past four years. I want to first thank Chas who helped me do my very first experiments and Kirsty both for her technical help and for being a such nice person to share a lab bench with for the past few years. I also want to thank the other students of the lab group for their help, kindness and encouragement over the past four years – Nina, Hamimah, Patoo, Varinitp, Cin, Adib and Rose. Sharing a group and bench with you all really kept me going.

I also want to thank Cosmin, Robin, Kay, Beth, Ricky and Abi of the Stevens group who I loved sharing a bay with. Thank you for your PhD advice, troubleshooting and conversations – I appreciated it so much. Many thanks also to Mark Stevens for sharing strains and *Salmonella* advice over the past four years and thank you to Dominic Kurian for taking me through the protein digestion process and performing the shotgun proteomics.

Thank you to the EASTBIO DTP both for funding my project and for funding a six-month extension while the COVID-19 pandemic extinguished my hopes of finishing my thesis and laboratory experiments on time. I also want to thank EASTBIO for encouraging a three-month internship. My placement at NIBSC was a truly wonderful experience which challenged my imposter syndrome and made me a better scientist and person. Thank you especially to my mentor Jun Wheeler for her patience while working with a biologist.

Of all my friends in Edinburgh I want to first thank my flatmate Rosie so much for being the best person to live with. I also want to thank Ciara, Jess, Agata, Marta, James, Izzie, Omar, Amanda, Rose, Abraham, Alba, Deepti, Fiona, Izzy, Emily, Becky, Susi and countless others for all the fun, pot-lucks, walking adventures and commiserations over the years.

My friends dotted about the world – Ines, Amy, Olivia, Skye, Kami, Adam and Rachel – thank you so much for being the best wee group.

Finally, I want to thank my family. My grandparents Rena and Archie and Jean and Joe have been such a huge part of my life and I want to thank them for their support. I want to also thank my aunt Claire and cousin Keir who took the time to help me with my lay summary. Finally, my mum and dad, Su and David, I don't know where I would be without you. Your love and support over the past 27 years means the world to me and this thesis is dedicated to you.

Contents

List of figures

List of tables

Chapter 1 Introduction

1.1 *Salmonella enterica*, a zoonotic pathogen of global importance

The first description of *Salmonella* bacteria was by German pathologist Karl Joseph Eberth who identified the bacillus in the spleen and abdominal lymph nodes of a typhoid fever patient in 1880 (Eberth, 1880). The bacteria, first titled *Eberthella*, was eventually named *Salmonella* after veterinary pathologist Daniel Elmer Salmon who, with fellow bacteriologist Theobald Smith, attributed fatal disease of pigs to the same bacillus discovered by Eberth (Smith, 1884; Nomenclature Committee of the International Society for Microbiology, 1934).

In 1987 *Salmonella enterica* was thus divided into seven subspecies – *Salmonella enterica* subsp. *enterica, Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *houtenae, Salmonella enterica* subsp. *indica, Salmonella enterica* subsp. *salamae* and *Salmonella enterica* subsp. *diarizonae* (Le Minor and Popoff, 1987; Penner, 1988). The work in this thesis, unless otherwise specified, refers to *S. enterica* subspecies *enterica* as *S. enterica*.

The now infamous causative agent of foodborne disease in humans, *S. enterica* are Gram-negative rod-shaped bacteria which are both facultative anaerobes and facultative intracellular organisms. The past 140 years of research have yielded a rich understanding of *Salmonella* evolution and pathogenesis but importantly has also identified many unanswered questions worthy of investigation.

Whilst *Salmonella* disease manifestation can vary from enteritis to enteric fever depending on the infecting *S. enterica* serovar and the immune status of the host, salmonellosis is most commonly associated with self-limiting gastroenteritis in healthy individuals. Typical disease presentation is depicted in Figure 1.1.

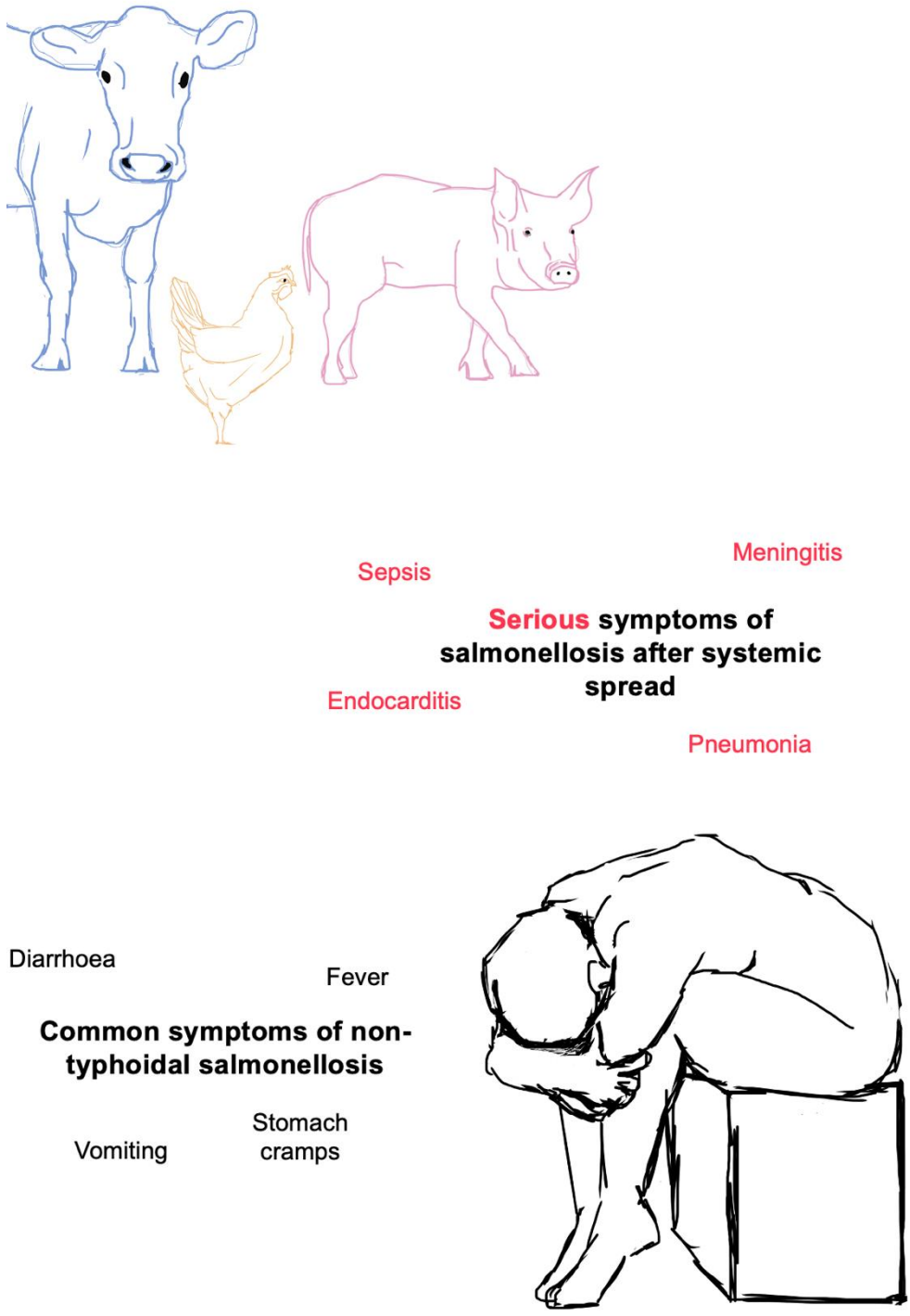


Figure 1.1 | Disease presentation of human salmonellosis.

Salmonellosis is usually foodborne and the result of livestock contamination. The common symptoms in immunocompetent humans are diarrhoea, fever, stomach cramps and vomiting. Immunocompromised and a small percentage of immunocompetent people suffer from severe disease resulting from systemic dissemination of *Salmonella* bacteria from the intestines to other organs.

Nearly 150 years after its identification, *S. enterica* remains a substantial health and socio-economic burden worldwide. There were 88 million cases, 123,000 deaths and 9 million disability adjusted life years (DALYs) estimated globally by the World Health Organisation in 2010 (Havelaar *et al.*, 2015). These figures, despite predicted to be an underestimation, highlighted an often-ignored result of infectious disease – DALYs – which have long term health and financial impacts.

Infections can cause long-term sequelae in multiple organs, even among healthy immunocompetent individuals, which can arise months after infection (Batz, Henke and Kowalcyk, 2013) such as chronic diarrhoea, postinfectious irritable bowel syndrome and reactive arthritis (Leirisalo-Repo *et al.*, 1997; Mearin *et al.*, 2005). The non-comprehensive list of serious symptoms of systemic salmonellosis described in Figure 1.1 have long term effects and can be life-altering (Cheng *et al.*, 2016; Thompson Bastin *et al.*, 2016). For immunocompetent patients, systemic spread occurs in around 5% of cases (Mandal and Brennand, 1988). The economic impact of *S. enterica* infections is therefore difficult to calculate – the costs of acute and chronic illness and agricultural and trade losses are expensive but rarely registered.

The largest recorded outbreak of salmonellosis in the Netherlands in 2013 resulted in 21,000 cases and four deaths with the causative agent identified as contaminated smoked salmon imported from Greece (Friesema *et al.*, 2014). The cost to the Netherlands was estimated at €7.5 million (Suijkerbuijk *et al.*, 2017) and despite the high expense the calculation did not include the additional losses related to the destruction of contaminated fish in Greece (Friesema *et al.*, 2014). As an example, the outbreak illustrates the exceptional dangers related to the zoonotic potential of *Salmonella* bacteria.

An astounding 86% of human infections are considered foodborne, derived from ingestion of contaminated animal products (Majowicz *et al.*, 2010) with the European Food Safety Authority (EFSA) naming *S. enterica* as the most common cause of foodborne infection in 2017 (EFSA, 2018).

Livestock, as a source of nutrition and environmental contamination, are therefore an important reservoir for human salmonellosis. Consumption of pork meat, for example, is a major risk factor for *S. enterica* disease globally (Campos *et al.*, 2019) and in eight countries – Belgium, Cyprus, Finland, France, Ireland, Italy, Poland and Sweden – intensively farmed pigs pose a higher risk for human infection than poultry (De Knegt *et al.,* 2015).

The subspecies *S. enterica* is further separated into over 2,500 serovars differentiated by serological typing. Serovars are defined by antigenic classification of the somatic O antigens, flagellar H antigens and Vi capsular antigens by the Kaufmann-White scheme, (Nomenclature Committee of the International Society for Microbiology, 1934; Ryan *et al.,* 2017) and additionally, adaptation to specific hosts (Uzzau *et al.*, 2000). There are 46 unique O groups (Liu *et al.*, 2014) and 114 unique H antigens (McQuiston *et al.*, 2004) whilst the Vi capsule is either present or absent (Pickard *et al.*, 2003; Faucher *et al.,* 2005). The most common serovars associated with foodborne outbreaks are readily found in intensely-farmed pig populations – *S. enterica* serovar Typhimurium (*S.* Typhimurium), *S.* Derby and *S.* Infantis (EFSA, 2019).

Pigs are often asymptomatic reservoirs of *S. enterica* with vertical sow-to-piglet and horizontal environment-to-pig transmission occurring on commercial farms (Campos *et al.*, 2019). *S. enterica* can persist in the tonsils, gut and gut-associated lymphoid tissue (GALT) of healthy asymptomatic carriers which, when the animals are slaughtered, provides a large risk to human health (reviewed by (Bonardi, 2017)). At slaughter the prevalence of *S. enterica* varies globally from 12.7% in the EU (EFSA, 2018) to 14-40% in Africa (Akoachere *et al.*, 2009; Kikuvi, Ombui and Mitema, 2010) and 29-100% in Asia (Ma *et al.*, 2017; Trongjit *et al.*, 2017). Since control measures on commercial and domestic pig farming are regulated at the national level only (Campos *et al.*, 2019), the true global prevalence of *S. enterica* in swine could be much higher.

Whilst infections of *S. enterica* in pigs are often asymptomatic, clinical illness can still occur. Diarrhoeal disease caused by *S. enterica* serovar Typhimurium is usually self-limiting in pigs as it is in humans (Paulin *et al.*, 2007). Invasive disease is usually caused by *S. enterica* serovars such as *S.* Choleraesuis or *S.* Typhisuis which disseminate outwith the gut to other organs to cause disease with a high mortality rate (Uzzau *et al.*, 2000; Longo *et al.*, 2019). Whilst there is a low prevalence of *S.* Choleraesuis infections in the EU, the serovar is frequently isolated in Asia from invasive infections of pigs and humans (Chiu *et al.,* 2004; Wang *et al.*, 2006; Ferstl *et al.*, 2017).

Since antimicrobial drugs are critical for treating severe salmonellosis in humans and suffering livestock, antimicrobial resistance in *Salmonella* bacteria is a growing public health concern. A multidrug resistant (MDR) *S.* Typhimurium (strain DT104) which arose in the 1960s is now globally associated with pig farming (Threlfall, 2000). It cannot be treated with ampicillin, chloramphenicol, streptomycin, sulphonamides or tetracycline (Antunes *et al.*, 2011; Gomes-Neves *et al.*, 2014) and has the potential to be transferred to human populations by infected meat.

Without mass vaccination programmes and with no broad-spectrum commercial vaccine currently in use, the vertical transmission in large piggeries leads to a perpetual reservoir of *Salmonella.* In outbreaks of clinical disease caused by *S.* Choleraesuis rudimentary killed vaccines have been employed to protect animals but killed *Salmonella* vaccines are not considered commercially viable since they may affect prevalence testing at slaughter (Wales and Davies, 2017).

There have been a variety of *S.* Typhimurium and *S.* Choleraesuis commercial vaccines available since the 1960s – ‘Suscovax’, ‘Salmoporc STM’ and ‘Salmoporc SCS’ and ‘Enterisol SC-54’. Each vaccine has been shown to confer protection against the specific serovar the vaccine was derived from (Hanna *et al.,* 1979a; Hanna *et al.,* 1979b; Roof and Doitchinoff, 1995; Eddicks *et al.*, 2009). Several cross-protection studies found that

vaccinating with live attenuated *S.* Choleraesuis vaccines awarded partial protection against *S.* Derby colonisation (Groninga *et al.*, 2000) and significant reduction in clinical symptoms but not colonisation by *S.* Typhimurium (Charles *et al.*, 2000; Husa *et al.*, 2009). Differences in the antigenic formulae that distinguish serovars provides a substantial challenge for vaccine design which protect against both colonisation and disease.

In this thesis, the two serovars of great interest are *S.* Typhimurium and *S.* Choleraesuis. Both, as previously mentioned, cause serious infections in swine and in humans but result in distinct clinical syndromes. The molecular mechanisms controlling the differential virulence have yet to be understood.

1.2 *S. enterica* serovars

Whilst there are over 2,500 serovars serologically typed, the range of hosts infected by serovars is the most clinically relevant definition.

Table 1.1 details a non-comprehensive list of important serovars which differ by natural host range and disease.

Table 1.1 | The host range of several important *S. enterica* serovars.

Host-generalist serovars cause self-limiting enteritis in a large range of hosts; host-adapted serovars infect few hosts and cause systemic disease; and host-restricted serovars have one natural host and cause systemic disease.

Host range	Serovar	Natural hosts and disease
Host-generalist	*S.* Enteritidis	Cattle, swine, humans, poultry – enteritis
	S. Typhimurium	Cattle, swine, humans, poultry – enteritis
Host-adapted	*S.* Choleraesuis	Swine – septicemia, chronic wasting, abortion, pneumonia Humans – septicemia, pneumonia
	S. Dublin	Cattle – enteritis, septicemia, abortion Humans – enteritis, septicemia Sheep – abortion
Host-restricted	*S.* Abortusovis	Sheep – abortion, septicemia
	S. Gallinarum	Poultry – septicemia, enteritis
	S. Typhi	Humans – septicemia
	S. Typhisuis	Swine – septicemia

Table 1.1 illustrates the disease manifestations of several important host-generalist, host-adapted and host-restricted serovars in their natural hosts. There is a distinct relationship between increasing host-restriction and systemic spread.

Interest in host-adaptation of *Salmonella* is over 120 years old, first discussed by pioneering bacteriologist Theobald Smith in his 1900 publication:

> *"It then becomes evident that adaptation to certain hosts has restricted their virulence more or less to these hosts. A variety which produces disease among swine may be harmless to cattle. … To admit that pathogenic forms can arise in short spaces of time is to greatly underrate the problem of parasitism and to overlook the existence of a complex relationship between host and parasite, because we cannot comprehend it."* (Smith, 1900)

Host-adapted serovars can have multiple natural hosts but, like host-restricted serovars, cause septicaemic disease which is often fatal (Jones *et al.*, 2008). Serovars such as *S.* Choleraesuis and *S.* Dublin therefore pose a greatly concerning zoonotic health risk in humans.

The differential manifestation of *S.* Typhimurium or *S.* Choleraesuis disease thereby correlates with host range. *S.* Typhimurium is a ubiquitous host-generalist which causes acute enteritis in many host species whilst *S.* Choleraesuis infection results in severe systemic disease in two known natural hosts.

In the next section, the pathology of host-generalist, host-restricted and host-adapted *Salmonella* infections are described.

1.2.1 Host-generalist *S. enterica*

Host-generalist serovars are the most richly-studied *S. enterica* pathogens due to their wide-host range and zoonotic risk. Most of the well-studied *S. enterica* serovars are ubiquitous host-generalists and capable of causing

outbreaks of foodborne enteritis which easily spreads between humans. Recently for example, there was a multi-country outbreak of *S.* Enteritidis linked to poultry produced in Poland (EFSA & ECDC, 2021) and a multi-country outbreak of *S.* Agona from an unknown source (EFSA & ECDC, 2018).

S. Typhimurium is a well-studied pathogen of warm-blooded animals and causes self-limiting enteritis in most cases. The serovar is a common cause of the acute foodborne enteritis depicted in Figure 1.1 and disease resolves within 7 days without the requirement of antibiotic treatment. In livestock animals such as pigs, cattle and chickens *S.* Typhimurium disease is restricted to the gut but severe systemic symptoms can develop in young animals (Barrow *et al.*, 1987), requiring antibiotic treatment and veterinary care.

Whilst *S.* Typhimurium is referred to as a host-generalist which causes acute enteritis within this thesis unless otherwise stated, it must be noted that some strains of *S.* Typhimurium do cause invasive systemic disease. *S.* Typhimurium sequence type 313 (ST313) for example, causes serious systemic invasive disease in sub-Saharan Africa (Reddy *et al.,* 2010), with its virulence potentiated by co-infection with malaria and HIV (Feasey *et al.*, 2012). Confounding publications have reflected on the ability of ST313 to infect other hosts, suggesting that ST313 is not restricted to humans (Parsons *et al.*, 2013) while another report remarked that the genome of multiple ST313 strains demonstrated signatures of host-restriction (Okoro *et al.*, 2015). A second example of *S.* Typhimurium host-restriction is U288, a variant which is dominant on UK pig farms but rarely causes human disease (Mueller-Doblies *et al.,* 2013), possibly due to its persistence in systemic sites or sensitivity to desiccation (Kirkwood *et al.*, 2021).

S. Typhimurium is one of the most well studied organisms and is often treated as a model host-generalist with molecular mechanisms controlling

bacterial colonisation and enteropathogenesis exceedingly studied in multiple host species compared to most host-restricted or -adapted serovars.

1.2.2 Host-restricted *S. enterica*

Serovars restricted to a single natural host are sharply juxtaposed from host-generalists.

S. Typhi, *S.* Sendai and *S.* Paratyphi A are restricted to humans and cause 9 million cases and 64,000 deaths estimated by the WHO in 2015 (Havelaar *et al.*, 2015). The disease, as detailed in Table 1.1, is systemic with bacterial dissemination to the blood, liver, spleen and bone marrow with little enteric symptoms (Dougan and Baker, 2014). A 14 day incubation period, high fever, vomiting, headache and increased heartrate characterise typhoid disease (Parry *et al.*, 2002; Olsen *et al.*, 2003), a strikingly different clinical syndrome than *S.* Typhimurium salmonellosis (Figure 1.1).

S. Gallinarum causes systemic fowl typhoid with natural disease restricted to the poultry host (Chadfield *et al.*, 2003). Infected birds suffer from enteritis, respiratory distress and swelling of the infected kidneys, spleen and liver and mortality can be up to 90% (Barrow *et al.*, 1987; Audisio and Terzolo, 2002). In contrast with *S.* Typhi which cannot survive in the non-human host (Spanò and Galán, 2012), *S.* Gallinarum, can still experimentally infect cattle and escape from the intestinal lumen to the lymphatics, although asymptomatically (Paulin *et al.*, 2002).

Sheep-restricted *S.* Abortusovis is a major cause of abortions and lamb death in flocks globally (Pardon *et al.*, 1988). Despite the main symptom of infection being abortion in ewes, the bacteria can be isolated in high numbers from other systemic organs – the bloodstream, liver, spleen, brain and stomach (Sanchis *et al.,* 1995), like other host-restricted serovars.

S. Typhi is the most well-studied host-restricted serovar with the discovery of specific interactions between bacterial virulence factors and the host

defences preventing *S.* Typhi bacteria from surviving in non-human hosts (Spanò and Galán, 2012; Spano *et al.*, 2016; Solano-Collado *et al.*, 2021).

1.2.3 Host-adapted *S. enterica*

Host-adapted serovars have several hosts, like host-generalists, but cause systemic disease much like host-restricted pathogens.

S. Choleraesuis shares its antigenic formula with similarly swine-restricted *S.* Typhisuis and human-restricted pathogen *S.* Paratyphi C (Uzzau *et al.,* 1999) and has a high mortality rate in swine and humans (Chiu *et al.,* 2004; Chen *et al.*, 2007). Porcine infections result in septicaemia, fever, pneumonia, chronic wasting and abortion with little enteric inflammation (Chiu *et al.,* 2004; Paulin *et al.*, 2007). In humans, the serovar also disseminates and colonises the bloodstream, bones and liver but can additionally cause pneumonia and endocarditis (Wang *et al.*, 2006; Chen *et al.*, 2007).

S. Dublin, too, is systemically virulent in cattle, humans and sheep but is mainly isolated from the bovine host (Harvey *et al.*, 2017). It causes fever, enteritis, septicaemia and abortions. Distinct from *S.* Choleraesuis, enteritis is a common *S.* Dublin infection symptom. *S.* Dublin contamination of ground beef recently caused a multi-state outbreak of salmonellosis in the US and resulted in thirteen cases, nine hospitalisations and one death (CDC, 2019).

A dated but comprehensive ten-year study of hospitalisations in the US due to human salmonellosis uncovered that infection with *S.* Dublin had the highest mortality rate and that cases of *S.* Choleraesuis and *S.* Dublin were the most likely to result in hospitalisation (Jones *et al.*, 2008). More recent data from the United States illustrated that there are between 10-20 *S.* Choleraesuis and an estimated 150 *S.* Dublin confirmed infections per year (CDC, 2018) which demonstrated that host-adapted serovars are still a public health concern.

1.2.4 *S.* Typhimurium and *S.* Choleraesuis in the porcine host

Evidently, host-generalist and host-adapted serovars cause unique disease presentations in the same host. *S.* Typhimurium and *S.* Choleraesuis both cause disease in swine and in humans but *S.* Typhimurium is restricted to self-limiting enteritis whilst *S.* Choleraesuis causes systemic disease.

An important *in vivo* comparative study involving both oral inoculation and ligated ileal loops detailed the pathological differences between infections specifically in the porcine host (Paulin *et al.*, 2007). The results are summarised in Figure 1.2.

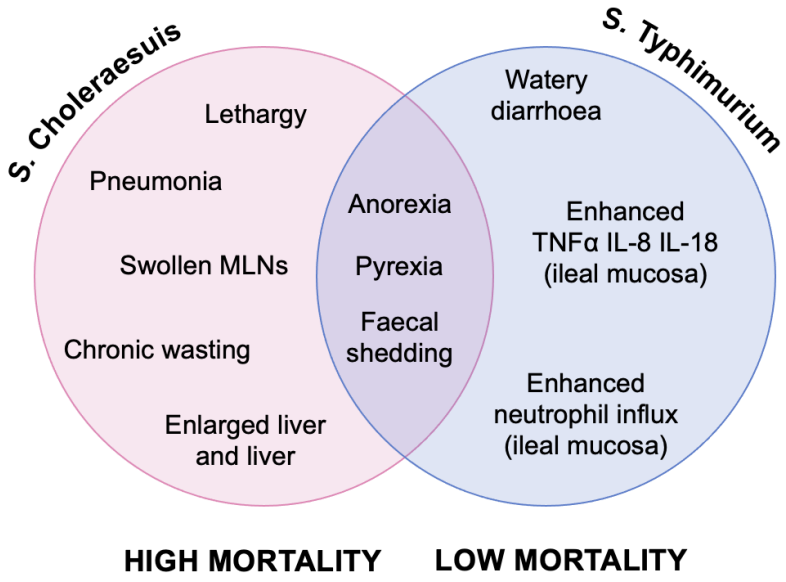


Figure 1.2 | Differences in *S*. Typhimurium and *S*. Choleraesuis pathological disease in *swine*.

Data collected from an *in vivo* study identified distinct clinical disease caused by the two serovars (Paulin *et al.*, 2007).

The authors discovered that *S.* Choleraesuis invaded the mucosa of ligated ileal loops less than *S.* Typhimurium and also induced significantly less fluid secretion and recruitment of neutrophils to the infected ileum (Paulin *et al.*, 2007). The systemic dissemination and high mortality of *S.* Choleraesuis bacterial infection is evidently not due to extreme intestinal pathology.

In the same study, oral infections demonstrated that *S.* Choleraesuis were isolated in greater numbers than *S.* Typhimurium from the ileal, colonic and mesenteric lymph nodes but less in the ileal mucosa (Paulin *et al.*, 2007). By measuring replication of the serovars after both oral and ligated loop infection, it was established that *S.* Typhimurium pathology in pigs is linked to its rapid replication in the intestinal mucosa which induces a significant inflammatory response leading to enteritis. *S.* Choleraesuis, conversely, replicates slowly in the ileal mucosa, induces little inflammation and persists in the mesenteric lymph nodes (Paulin *et al.*, 2007). This result was corroborated by other researchers studying the porcine immune response against both serovars (Uthe *et al.*, 2007).

Promising subsequent experiments in culture and in porcine alveolar macrophages also illustrated that *S.* Typhimurium transcribed and secreted more *Salmonella* invasion protein C (SipC) than *S.* Choleraesuis which was a pioneering mechanistic observation (Paulin *et al.*, 2007).

Overall, the comparative and comprehensive infection study discovered important pathological differences between the serovars. The molecular basis of these differences during pathogenesis, however, remains poorly understood.

1.3 *S. enterica* pathogenesis

Regardless of infecting serovar and outcome of infection, passage through the gastrointestinal tract, colonisation of the intestines and evasion of the host immune response is crucial for *S. enterica* disease.

Most research has utilised the model host-generalist pathogen *S.* Typhimurium as the infectious agent and therefore unless otherwise stated, the molecular mechanisms detailed in this section were uncovered using this serovar. The genetic and pathological diversity among non-typhoidal and typhoidal serovars, however, suggests that what is true for *S.* Typhimurium may not be true for all serovars (Paulin *et al.*, 2007; Baldassarre *et al.*, 2021). The use of particular strains must also be considered when reviewing *Salmonella* pathogenesis. The LT2 strain has been used as a model organism since the 1940s in cellular, animal, genetic, transcriptomic and proteomic experiments but, due to laboratory passage, was later discovered to encode a non-functional sigma factor *rpoS,* rendering stocks of LT2 avirulent (*Swords et al.,* 1997).

Most experimental studies of bacterial virulence, with few exceptions, were performed using gene deletion mutants in either cells or mice. Whilst mice are a useful model host organism due to their low expense, *S.* Typhimurium disease in the murine host is more akin to human typhoid fever hence *S. enterica* serovar 'Typhi'-'murium' (Nomenclature Committee of the International Society for Microbiology, 1934). To use mice as a model for enteropathogenesis, the bactericidal antibiotic streptomycin is used to change the composition of the murine intestinal microbiota (Barthel *et al.*, 2003) and disrupt the dynamic interactions between invading *Salmonella* and commensal bacteria which have been shown to be key to *Salmonella* pathogenesis (Thiennimitr *et al.*, 2011; Drumo *et al.*, 2016; Argüello *et al.*, 2018). The developed streptomycin-treated model is thus useful for the study of enteropathogenesis but lacks an important component of host defence.

Several multi-host studies of the *S.* Typhimurium genes involved in pathogenesis additionally uncovered that different bacterial factors are essential in different hosts (Tsolis *et al.*, 1999; Wallis and Galyov, 2000; Morgan *et al.*, 2004; Chaudhuri *et al.*, 2013). The same caution applied to the use of specific serovars must therefore be applied to the use of specific hosts in research.

The next three sections detail the stages of *Salmonella* infection from oral ingestion to symptomatic disease.

1.3.1 The early stages of *S. enterica* infection

Salmonellosis begins with oral ingestion of *S. enterica* bacteria, potentially as few as 100 cells (Waterman and Small, 1998; Gawande and Bhagwat, 2002), which reach their favoured intestinal niche by avoiding the anti-bacterial nature of the gastrointestinal tract. The stomach is one of the first barriers against bacterial colonisation with a pH as low as 1.5. Several bacterial strategies, however, compound the stomach's hostile anti-bacterial nature. Sensing and responding to environmental conditions is crucial both for *S. enterica* virulence and for survival within extreme settings – the acid tolerance response (ATR) (Foster and Hall, 1990) allows *S. enterica* to adapt to the gastric environment and is necessary for bacterial survival within bone-marrow derived macrophages (Wilmes-Riesenberg *et al.*, 1996).

The ATR has been investigated in a range of *S. enterica* serovars including both host-generalist and host-restricted pathogens with poorer *in vitro* survival of host-restricted *S.* Typhi after acid shock compared to *S* Typhimurium identified (Tiwari *et al.*, 2004). The risk of foodborne salmonellosis is emphasised by the inference that more bacteria survive the stomach acidity when consumed with food, and therefore exposure to fewer bacteria results in gastrointestinal colonisation and disease (Rychlik and Barrow, 2005). It is clear that before *S. enterica* reaches the intestinal mucosa, a multitude of genes not related to invasion and intracellular survival are critical.

Swimming motility is an important virulence factor in the early stages of infection. Motility is conferred by the presence of 5-10 peritrichous flagella on the bacterial cell surface which are expressed by most serovars and composed of the protein flagellin. Some serovars encode two genes which encode flagellin – *fliC* and *fljB* – but many have lost *fljB* during evolution (McQuiston *et al.*, 2008). *S.* Dublin and *S.* Choleraesuis, for example, only

encode *fliC* (McQuiston *et al.*, 2008) and therefore cannot exhibit phase variation (Kutsukake and Iino, 1980). With the exception of host-restricted poultry pathogens *S.* Gallinarum and *S.* Pullorum (Foley *et al.*, 2013), most serovars are motile.

Flagella rotate and allow bacteria to navigate the gastrointestinal tract and intestinal mucosa driven by chemical signals. The appendage binds actin and host cell lipids to adhere and promote invasion of epithelial cells (Wolfson *et al.*, 2020). After bacterial invasion, flagella are recognised by the intracellular domain of toll-like receptor 5 (TLR-5) on the basolateral surface of the intestinal epithelium (Hayashi *et al.*, 2001) and induce the secretion of pro-inflammatory cytokines (Gewirtz *et al.*, 2001).

Differences in the inflammatory response induced at the ileal mucosa distinguish *S.* Typhimurium and *S.* Choleraesuis disease *in vivo* (Paulin *et al.*, 2007) which could be influenced by flagella. The reliance on flagella for virulence in epithelial cells and macrophages by *S.* Typhimurium and host-adapted *S.* Dublin has been published (Olsen *et al.*, 2013). Deletion of *fliC* similarly attenuated invasion of epithelial cells and increased bacterial survival within macrophages of both serovars (Olsen *et al.*, 2013). The study validated the importance of the flagella to both serovars but also noted that more studies are needed to unpick serovar differences in appropriate animal models.

Also at the intestinal mucosa, the aforementioned commensal microbiota provide both competition but also nutritional carbon sources for invading *Salmonella* (Faber *et al.*, 2017).

Pigs whose *S.* Typhimurium subclinical infection resulted in a low level of faecal shedding had a different gut microbiota composition compared to clinically infected high level shedders (Bearson *et al.*, 2013; Drumo *et al.*, 2016). Since less *S.* Choleraesuis than *S.* Typhimurium was shed from infected pigs after oral infection by Paulin and colleagues (Paulin *et al.*,

2007), the complex interactions between the microbiota and *Salmonella* must be remarked upon. The composition changes are most likely a result of inflammation (Stecher *et al.*, 2007) but could also be due to differences in anaerobic metabolism between the serovars (Thiennimitr *et al.*, 2011; Nuccio and Bäumler, 2014). Exploring links between bacterial replication, the gut microbiota and specific serovars could give a greater insight into the relevance of metabolism and inflammation to disease outcome.

Mucus covers the apical side of the intestinal epithelium, secreted by goblet cells present in the epithelium, and is composed of aggregated mucin glycoproteins, IgA antibodies and anti-microbial peptides (Patel and McCormick, 2014). The mucus functions as a physical and immune barrier to both invading pathogens and the commensal microbiota from the epithelium and, if secreted from cells, act as receptor decoys which bacteria bind (Lindén *et al.*, 2009). *S. enterica,* however, uses the overlaying mucus layer to its advantage, binding the 460 kDa transmembrane mucin MUC1 (Li *et al.*, 2019) and the 250 kDa neutral mucin (Vimal *et al.*, 2000). The transmembrane MUC1 is anchored to cells at mucosal surfaces, extending ~500 nm from cell surfaces (Van Putten and Strijbis, 2017) and is involved in host signal transduction pathways including the promotion of inflammation (Ng *et al.*, 2016).

The *Salmonella* giant adhesin SiiE, which is secreted by a type 1 secretion system (T1SS), binds MUC1 to adhere bacteria to epithelial cells and aid invasion (Li *et al.*, 2019). The 595 kDa adhesin is secreted by the *Salmonella* Pathogenicity Island 4 (SPI-4) T1SS and is important for the invasion of polarised epithelial cells (Gerlach *et al.*, 2007, 2008) and the colonisation of cattle and mice but remarkably not chickens or pigs (Morgan *et al.*, 2004, 2007; Carnell *et al.*, 2007).

Fimbriae are an additional large class of cell surface adhesins encoded by both typhoidal and non-typhoidal *S. enterica* serovars (Althouse *et al.*, 2003). Type IV fimbriae are an example of fimbriae only encoded by *S.* Typhi, *S.*

Paratyphi C and isolates of *S.* Dublin positive for SPI-7 (Morris *et al.*, 2003) and allow the serovars to use the cystic fibrosis transmembrane conductance regulator (CFTR) as a receptor (Tsui *et al.*, 2003). Fimbriae are interesting adhesins because of their genetic diversity across serovars. For example, there are more non-functional fimbrial genes in host-restricted and host-adapted than host-generalists (Yue *et al.*, 2012, 2015) suggesting that some fimbrial genes are host-specific.

In contact with the intestinal epithelium, adhered by the flagella, fimbriae or SiiE, *S. enterica* expresses the type III secretion system (T3SS) encoded on SPI-1 (T3SS-1) which is a needle-like molecular syringe (Lee, Jones and Falkow, 1992). While the T3SS-1 is the most well-studied *Salmonella* invasion strategy there are several bacterial factors that also aid internalisation including resistance to complement killing (Rck) and PhoP activated gene N (PagN) proteins (Lambert and Smith, 2008; Rosselin *et al.*, 2010; Roche *et al.*, 2018).

The T3SS forms a pore in the host membrane to inject effector proteins, including invasion protein SipC, from the bacterial cytoplasm to the host cytosol which promote bacterial internalisation. The triple-membrane spanning structure is depicted in Figure 1.3, *in situ* (Figure 1.3A) and in association with the host (Figure 1.3B).

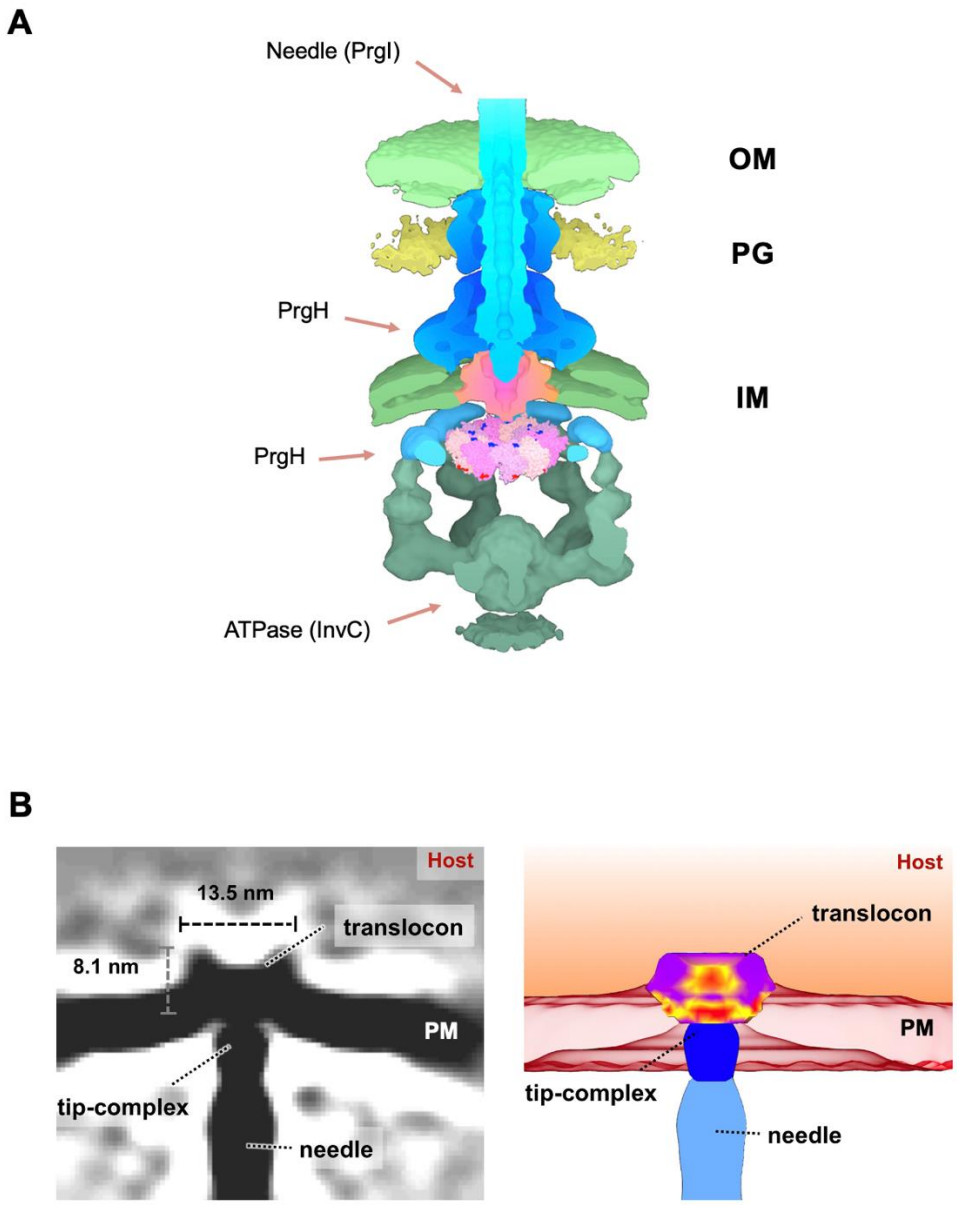


Figure 1.3 | Architecture of the *S. enterica* T3SS-1 *in situ* and in contact with host cells.

(A) The structure of the T3SS-1 injecitsome as defined by cryo-electron tomography and sub-tomogram averaging, adapted from Hu *et al.,* (2017) (licenced by Copyright Clearance Centre; License Number 5042971114074). The system spans the bacterial inner membrane (IM), peptidoglycan (PG) and outer membrane (OM).

(B) The intimate interaction of the T3SS with host cells defined by cryo-electron tomography, adapted from Park *et al.*, (2018) (Creative Commons Attribution Licence). The translocon is composed of effector proteins SipB, SipC and SipD and it makes physical contact with the host plasma membrane (PM).

Injection of T3SS-1 effectors from the bacterial cytoplasm through the PrgI needle (Figure 1.3) is important for colonisation of porcine intestines by both *S.* Typhimurium and *S.* Choleraesuis (Lichtensteiger and Vimr, 2003; Carnell *et al.*, 2007; Chaudhuri *et al.*, 2013).

Bacteria invade both the follicle-associated epithelium (FAE) and absorptive epithelium (Bolton *et al.*, 1999) of the distal ileum (Carter and Collins, 1974). Neither *S.* Typhimurium or *S.* Choleraesuis displays a preference for the specialised microfold (M) cells of the porcine FAE (Bolton *et al.*, 1999; Meyerholz and Stabel, 2003) which differs from the murine model of systemic salmonellosis which suggested that *S.* Typhimurium preferentially invades M cells (Jones *et al.,* 1994). M cells overlay lymphoid follicles called Peyer's patches, which are populated by macrophages and dendritic cells (Da Silva *et al.*, 2017). The invasion of M cells allows bacteria access to antigen-sampling dendritic cells which phagocytose bacteria to present antigens to naïve T and B cells in the mesenteric lymph nodes thereby increasing the potential for bacterial systemic dissemination (Allenspach *et al.*, 2008; McLaughlin *et al.*, 2009).

Both serovars induce morphological changes at the intestinal epithelium in porcine ligated ileal loops (Meyerholz and Stabel, 2003). Morphological changes are most likely a result of the intracellular activities of effector proteins since a comprehensive *in vivo* porcine gene expression study after the infection with both serovars noted upregulation of genes ARPC2, LCP1, SDCBP and HSPH1 which have annotated functions related to regulation of the cellular cytoskeleton (Uthe *et al.*, 2007).

Figure 1.4 depicts the morphological changes in the distal bovine ileum after challenge with *S.* Typhimurium and the T3SS effector proteins which influence bacterial invasion (Figure 1.4).

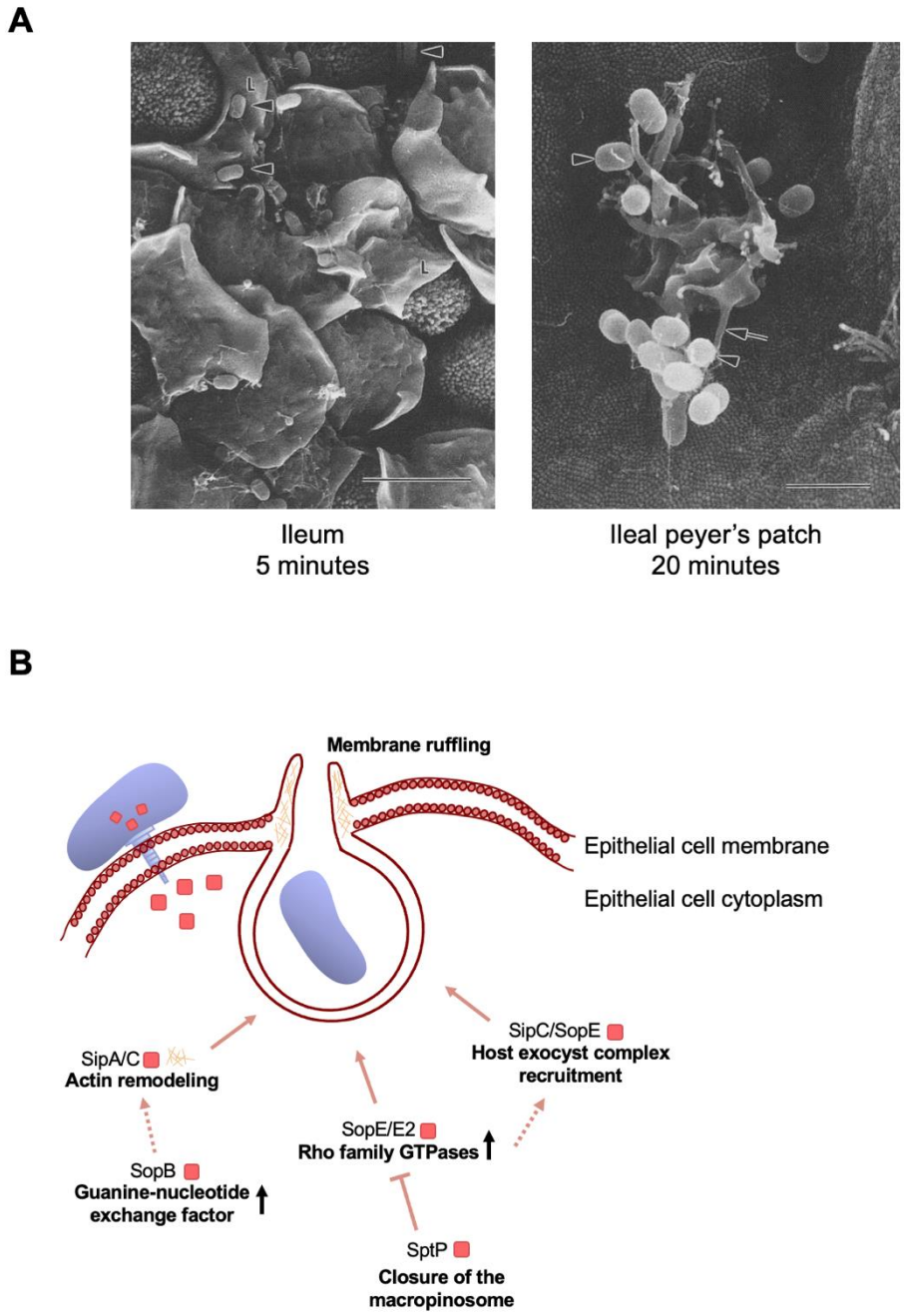


Figure 1.4 | *S. enterica* intestinal invasion.

(B) Scanning electron micrographs of *S.* Typhimurium bacteria at bovine ileal loops after challenge for 5 or 20 minutes. Adapted from Frost *et al.,* (1997), copyright © 1997, © SAGE Publications.

- After 5 minutes bacteria induced membrane ruffles (L) which the bacteria (arrow head) associate with. (Scale bar = 5 μm).
- At the Peyer's patch bacteria (arrow head) also adhere to membrane ruffles (arrow) (Scale bar = 10 μm).

(B) *S. enterica* facilitates the invasion of host cells by the secretion of T3SS effector proteins which manipulate the host cytoskeleton and biochemical signalling pathways.

Invasion of non-phagocytic cells of the intestinal epithelium requires the activities of T3SS-1 secreted effector proteins in concert which remodel actin and manipulate host cell signalling to promote bacterial invasion by macropinocytosis (Figure 1.4).

The formation of 'membrane ruffles' or lamellipodia, morphological extensions of the host plasma membrane around invading bacteria, is induced by the direct actin binding of T3SS-1 secreted effector proteins SipA and SipC. Figure 1.4 illustrates the membrane ruffles induced by invading *S.* Typhimurium in the bovine ileum which the bacteria associate with during invasion after just five minutes post-infection (Frost *et al.,* 1997). SipC bundles and nucleates actin via its C-terminal domain while SipA prevents actin from depolymerising to potentiate the activities of SipC (Zhou, 1999; Chang *et al.,* 2005; Chang *et al.*, 2007).

In addition to actin remodelling at the cell membrane, *Salmonella* also injects proteins which manipulate host cell signalling to promote membrane ruffling, internalisation of bacteria and destabilisation of tight junctions between cells. SopE and SopE2 mimic the guanine-nucleotide exchange factors (GEF) of the host to bind and activate Rho-GTPases RalA (SopE), Rac-1 (SopE), RhoG (SopE) and Cdc42 (SopE and SopE2) by catalysing the formation of GTP from GDP (Hardt *et al.*, 1998; Criss *et al.*, 2001; Friebel *et al.*, 2001; Patel and Galán, 2006). SopB, a phosphoinositide phosphatase, is also involved in membrane ruffling and internalisation of bacteria by activating a host GEF which activates host protein RhoG to promote membrane ruffling and engulfment (Patel and Galán, 2006).

The complex host-pathogen interactions at the intestinal epithelium also result in activation of the host immune system.

The Nuclear Factor κB (NF-κB), Signal Transducer and Activator of Transcription 3 (STAT3), and Mitogen Activated Protein (MAP) kinase pathways are activated by *S. enterica* virulence factors such as LPS, flagellin

and effector proteins (Gewirtz *et al.*, 2001). Epithelial cells and phagocytes express TLRs and nucleotide-binding and oligomerisation domain (NOD)-like receptors (NLRs) at the apical surface and on the intracellular basolateral membrane to recognise specific antigens before and after bacterial invasion (Chamaillard *et al.*, 2003; Chieppa *et al.*, 2006; Rydström and Wick, 2007).

NLRs are also a critical component of canonical and non-canonical inflammasome complexes, bound to either inactive caspase-1 (canonical) or caspase-4, -5 and -11 (non-canonical) (Storek and Monack, 2015). Once NLRs recognise specific antigens like flagellin and the T3SS-1 apparatus, caspases are cleaved and activated to trigger inflammatory cell death (Fink and Cookson, 2006).

NLR binding also activates the NF-κB pathway at the basolateral membrane and induces secretion of chemoattractant cytokines interleukin (IL)-8 and CCL20 to recruit PMNs and dendritic cells (Gewirtz *et al.*, 2000). After MAP kinase activation by the collected functions of SipA, SopB, SopE and SopE2, the dysregulation of tight junction integrity (Boyle *et al.,* 2006; Köhler *et al.*, 2007; Liu *et al.*, 2020) results in increased invasion, increased NF-κB signalling and increased IL-8 secretion (Yu *et al.*, 2003). Collective activation of the pathways and caspases lead to a cascade of pro-inflammatory cytokine production, inflammation and increased bacterial invasion from dying cells (Chen *et al.,* 1996; Hobbie *et al.*, 1997; Bruno *et al.*, 2009).

IL-8 secretion is a potent neutrophil chemoattractant and *in vivo,* was induced at significantly lower levels by *S.* Choleraesuis than *S.* Typhimurium in the porcine ileum (Paulin *et al.*, 2007). Reduced IL-8 secretion was also a differentiating characteristic of the response of human colonic cell line T84 cells to *S.* Typhi infection (Raffatellu *et al.*, 2005) and *S.* Paratyphi A infection of Caco-2 cells (Elhadad *et al.*, 2016). Additional cell line experiments also uncovered serovar-specific responses of porcine intestinal β-defensins – *S.* Typhimurium induced up-regulation whilst *S.* Choleraesuis had little effect (Veldhuizen *et al.*, 2009). For typhoidal serovars, reduced neutrophil

recruitment and intestinal inflammation is theorised to promote systemic spread (House *et al.*, 2001).

The cascade of cytokine signalling recruits phagocytes, polymorphonuclear neutrophils (PMNs), natural killer (NK) cells, dendritic cells and T cells to clear bacteria from the infected epithelium (Gewirtz *et al.*, 2001; Uthe *et al.*, 2007).

Whilst the recruitment of neutrophils to the infected epithelium is an innate anti-microbial strategy to clear *Salmonella* bacteria, it is also increased by the action of effector protein SipA which stimulates the release of neutrophil chemoattractant hepoxilin A3 (Wall *et al.*, 2007). *Salmonella* benefits from the rapid infiltration of neutrophils which also disrupts tight junctions, thereby increasing bacterial invasion (Köhler *et al.*, 2007).

To diminish the inflammatory response, T3SS-1 effectors AvrA and SptP are secreted to repair cytoskeletal rearrangements and stabilise tight junctions (Zhang *et al.*, 2015) by counteracting the activation of Rac-1 and Cdc42 (Fu and Galán, 1999) while proteases PipA, GtgA and GogA inhibit the NF-κB pathway (Sun *et al.*, 2016). The activities of these proteins allow the bacteria to intuitively control the host response to allow the retention of the preferential bacterial intracellular niche.

Inflammatory homeostasis between the host and bacteria develops. The bacteria induce inflammation which provides the key carbon sources to promote bacterial proliferation (Stecher *et al.*, 2007; Winter *et al.*, 2010). There are confounding arguments whether *S.* Typhimurium replicates faster than host-adapted serovars and therefore causes more inflammation (Paulin *et al.*, 2007) or whether *S.* Typhimurium induces more inflammation and can therefore proliferate faster.

Different host *in vivo* models have been used to investigate the invasion potential of serovars which differ by host range. Interestingly, however, increased invasion does not correlate with increased systemic spread of *S.*

Choleraesuis, *S.* Dublin, *S.* Gallinarum or *S.* Abortusovis in porcine, bovine, chicken and ovine models (Bolton *et al.*, 1999; Uzzau *et al.*, 2001; Paulin *et al.*, 2002, 2007; Chadfield *et al.*, 2003).

1.3.2 Bacterial intracellular survival after invasion of the intestinal epithelium

After initial invasion, *Salmonella* bacteria must form a complex relationship with the host to ensure their survival and proliferation.

Within the host cell *Salmonella* resides within a large intracellular *Salmonella*-containing vacuole (SCV), formed during macropinocytosis (Fredlund *et al.*, 2017) (Figure 1.5). The biogenesis of the SCV and development of an intracellular niche permissive to *Salmonella* replication is key to bacterial survival (Figure 1.5). The acidified environment of the early SCV induces the expression of the second T3SS (T3SS-2) encoded on SPI-2 (Shea *et al.*, 1996; Yu *et al.*, 2010) which secretes effectors to control host cell processing and subvert the host antimicrobial strategies.

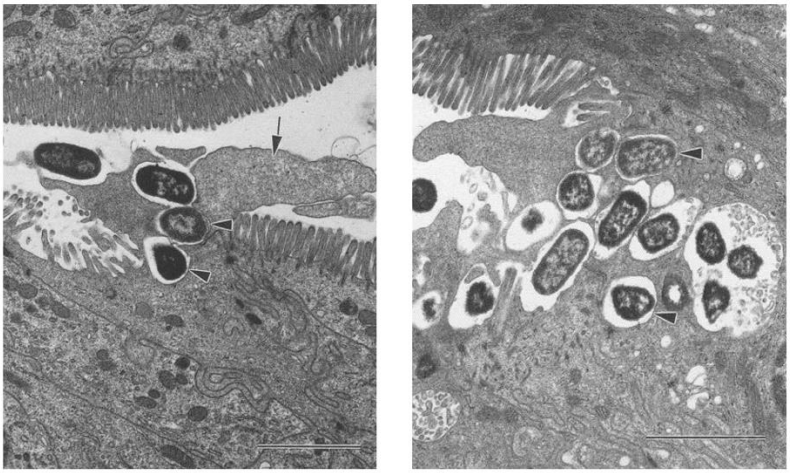


Figure 1.5 | Intracellular *S. enterica* reside within the SCV

Transmission electron micrographs of *S*. Typhimurium bacteria at the bovine ileal loops after challenge for 20 or 60 minutes. Adapted from Frost *et al.,* (1997), copyright © 1997, © SAGE Publications.

- After 20 minutes membrane ruffles are abundant (arrow) and the bacteria have been internalised in vacuoles (arrow head) (Scale bar = 2 μm).
- After 60 minutes many bacteria (arrow head) reside within vacuoles (arrow head) (Scale bar = 2 μm).

After engulfment the SCV immediately recruits host proteins EEA1, the transferrin receptor and Rab GTPase Rab5 which identify the vacuole for endocytic destruction (Méresse *et al.*, 1999; Steele-Mortimer *et al.*, 1999; Mukherjee *et al.*, 2000). Rab GTPases are critical regulators of the endocytic pathway that *Salmonella* manipulates (Prashar *et al.*, 2017). The SCV, whilst it retains phagosome markers, deviates from the model phagosome maturation pathway that would result in bacterial destruction.

T3SS-1 proteins SipA and SopB are still active early after invasion and have roles in SCV biogenesis and cellular localisation. SopB is critical for the recruitment of Rab5 to the vacuole (Hernandez *et al.*, 2004; Dukes *et al.*, 2006; Mallo *et al.*, 2008; Bakowski *et al.*, 2010) and SipA is involved in positioning the SCV close to the nucleus and recruiting lysosomal membrane protein 1 (LAMP1) to the vacuole membrane (Brawn *et al.,* 2007; Zhang *et al.*, 2018). LAMP1 acquisition is key to the acidification of the vacuole which stimulates *S. enterica* gene expression (Zhang *et al.*, 2018).

The pH of the SCV lumen lowers within two hours of maturation to 4.9 (Drecktrah *et al.*, 2007). This stimulates assembly of the needle-like T3SS-2 which secretes across the vacuole membrane into the host cytoplasm thereby facilitating bacterial survival and replication (Beuzón *et al.*, 1999; Yu *et al.*, 2010).

After losing markers EEA1 and the transferrin receptor, the next proteins recruited to the SCV are Rab7 and the vacuolar ATPase and LAMP1 (Garcia-Del Portillo *et al.,* 1995; Rathman *et al.,* 1997; Méresse *et al.*, 1999; Steele-Mortimer *et al.*, 1999).

Recruitment of Rab GTPases differs in vacuoles containing different *Salmonella* serovars. Specifically tested in murine epithelial cells and macrophages, the *S.* Typhi SCV recruits Rab32, Rab38 and Rab29 whilst *S.* Typhimurium does not (Spanò *et al.,* 2011; Spanò and Galán, 2012). The recruited GTPase Rab32 interacts with the Biogenesis of Lysosome-related

Organelle Complex (BLOC)-3 (BLOC-3) which leads to maturation of the phagosome and bacterial death (Baldassarre *et al.*, 2021). Two T3SS effectors absent from the genome of *S.* Typhi were identified as factors which control Rab GTPase decoration – GtgE and SopD2.

GtgE and SopD2 either cleave Rab29, Rab32 and Rab38 (protease GtgE) or induce the disassociation of Rab32 from the *S.* Typhimurium SCV (GTPase activating protein SopD2) (Spanò *et al.,* 2011; Spano *et al.*, 2016). Since human epithelial cells and macrophages also utilise Rab32/BLOC-3 as a defence against pathogens, the reason *S.* Typhi is able to evade destruction in the human host has not been fully explored (Baldassarre *et al.*, 2021). More studies are required to investigate differences in SCV maturation by other serovars and in different host species.

The late SCV (Figure 1.5) develops 3-4 hours after invasion and is surrounded by a network of tubular *Salmonella* induced filaments (Sifs) (Garcia-del Portillo *et al.*, 1993). Sifs, like the SCV itself are decorated with LAMP1 and promote nutrient acquisition, cell-to-cell transfer for bacteria within the vacuole and maintain the vacuolar membrane (Beuzon *et al.*, 2000; Szeto *et al.*, 2009; Liss *et al.*, 2017). T3SS-2 effectors that drive Sif formation are PipB2, SifA, SopD2, SseF and SseG (Stein *et al.*, 1996; Kuhle *et al.,* 2004; Knodler and Steele-Mortimer, 2005; Schroeder *et al.*, 2010).

Despite LAMP1 decoration on the late SCV and surrounding Sifs, the vacuole is protected from anti-microbial lysosomal enzymes and does not mature into a phagolysosome (Garcia-Del Portillo and Finlay, 1995; Rathman *et al.,* 1997). Protected from degradation, the late SCV is therefore permissive for bacterial proliferation.

In both epithelial cells and in macrophages effector SseG is secreted across the vacuolar membrane and controls the cellular localisation of the SCV to the Golgi body, a requirement for *Salmonella* replication within the vacuole (Hensel *et al.*, 1998; Salcedo and Holden, 2003). Furthermore, deletion of

sseG attenuates *S.* Typhimurium virulence in the murine model (Hensel *et al.*, 1998). SseF and SseG both assist the localisation of the SCV to the Golgi network and nucleus to promote bacterial vacuolar replication with adequate nutrition (Salcedo and Holden, 2003; Kuhle *et al.,* 2004; Deiwick *et al.*, 2006).

After the development of the intracellular replicative niche, the bacteria replicate within and outwith the vacuole (Knodler *et al.,* 2014). Whilst bacterial replication within the vacuole is led by T3SS-2 effectors, escape from the SCV and hyper-replication in the host cytosol is the result of a resurgence of T3SS-1 activity (Knodler *et al.,* 2014), suggesting that coordination between both T3SSs are key to bacterial intracellular survival.

After *Salmonella* successfully invades the intestinal epithelium, the bacteria can access the lamina propria. The lamina propria is a tissue rich in immune surveillant macrophages, NK cells, dendritic cells and T cells which control humoral and cell-mediated responses to bacterial infection (Patel and McCormick, 2014). *S.* Typhimurium usually colonises to the host intestines where it proliferates and induces the inflammatory response characteristic of gastroenteritis while host-restricted and adapted serovars spread systemically from the lamina propria.

1.3.3 *S. enterica* disease – enteritis and systemic dissemination

Self-limiting enteritis is the most common outcome of infection with host-generalist serovars such as *S.* Enteritidis and *S.* Typhimurium and is the result of substantial inflammation and fluid secretion into the intestinal lumen and colon.

As discussed in previous sections, invading and intracellular *S.* Typhimurium induces a strong immune response, which has typically been studied in cellular models of infection. Morphological changes, activation of TLRs and NLRs and manipulation of host cell processes result in inflammasome

activation, NF-κB pathway signalling and cytokine release similar to *in vivo* models.

In appropriate animal models of disease, the fluid secretion and recruitment of immune cells to infected tissues has been attributed to the actions of T3SS-1 effectors SopB, SopD and SopA (Galyov *et al.*, 1997; Jones *et al.*, 1998; Wood *et al.*, 1998, 2000a). SipA also has a role in neutrophil recruitment (Wall *et al.*, 2007). SopB is initially involved in host GEF signalling to promote invasion (Patel and Galán, 2006), but as a phosphoinositide phosphatase SopB activity results in increased intracellular hydrolysed inositol phosphates Ins(1,4,5,6)P4 levels which disrupt chloride channels and induce fluid secretion into the intestinal lumen (Norris *et al.*, 1998). Fluid secretion into the intestinal lumen leads to diarrhoea characteristic of salmonellosis (Norris *et al.*, 1998).

A robust immune response therefore eventually ends the symptomatic disease, with *Salmonella* shed in the faeces for over a month (Murase *et al.*, 2000).

Host-restricted and host-adapted serovars result in a markedly different clinical presentation of disease with little enteritis (Sprinz *et al.*, 1966). Systemic salmonellosis is characterised by escape from the gastrointestinal tract to colonise other organs such as the blood, joints, kidney, heart, liver or brain (Jones *et al.*, 2008).

For nearly 40 years survival within the macrophage has been considered critical for systemic spread of *Salmonella* bacteria (Fields *et al.*, 1986; Richter-Dahlfors *et al.,* 1997). Macrophages are professional phagocytes and reside in the intestinal lumen, lymphoid follicles and in the lamina propria (reviewed in (Viola and Boeckxstaens, 2020)). *Salmonella*, however, can survive after phagocytosis within SCVs (Uchiya *et al.*, 1999) and evade destruction by reactive oxygen intermediates nitric oxide and NADPH oxidase (Korshunov and Imlay, 2002). After evading macrophage

destruction, *S. enterica* can replicate in the blood, spleen, liver, kidneys and bone marrow (*Tam et al.,* 2008).

Oral challenge in swine with either serovar demonstrated that *S.* Choleraesuis was isolated in higher numbers in mesenteric lymph nodes (Paulin *et al.*, 2007) which suggested that increased survival within professional phagocytes during antigenic presentation could distinguish *S.* Choleraesuis from *S.* Typhimurium*. S.* Choleraesuis, however, did not survive better than *S.* Typhimurium in cultured primary porcine alveolar macrophages or induce different levels of pro-inflammatory cytokines (Watson *et al.*, 2000), suggesting that enhanced survival in phagocytic cells is not the mechanism *S.* Choleraesuis uses to disseminate outwith the intestinal mucosa.

Dendritic cells present in the Peyer's patch and in the lamina propria are considered of great importance for the dissemination of *Salmonella* (Patel and McCormick, 2014). Intracellular survival experiments with dendritic cells could give a greater insight than alveolar macrophages on the influence of phagocytes on *S.* Typhimurium and *S.* Choleraesuis disease.

The ability of an invasive *S.* Typhimurium isolate belonging to ST313 to cause systemic disease in humans, has been linked to the loss of the effector-encoding gene *sseI* by genomic decay (Carden *et al.*, 2017). SseI is a T3SS-2 translocated effector that reduces migration of macrophages and dendritic cells via IQGAP1 (McLaughlin *et al.*, 2009). In the 2017 study, a strain lacking *sseI* disseminated at a higher rate to mesenteric lymph nodes by invading migrating dendritic cells (Carden *et al.*, 2017).

The secreted *Salmonella* cytolethal distending toxin, also known as the typhoid toxin, is another bacterial virulence factor associated with invasive disease (Haghjoo and Galán, 2004) and passage across the blood brain barrier (Yang *et al.*, 2018). The toxin is carried by typhoidal serovars and at least 41 non-typhoidal serovars (den Bakker *et al.*, 2011) and is hypothesised

to contribute to the systemic spread of host-generalist *S.* Javiana (Miller *et al.*, 2018). It is not, however, encoded by all serovars which cause systemic disease.

In conclusion, reviewing the literature has emphasised the need for more study of host-adapted serovars of *Salmonella.* Swine in particular are an important reservoir for human salmonellosis and despite the risk of deadly porcine adapted *S.* Choleraesuis infection in humans, not enough is known about its pathogenesis.

1.4 Hypothesis and aims

Exploring the molecular mechanisms dividing *S.* Typhimurium and *S.* Choleraesuis disease pathogenesis will lead to a greater understanding of zoonotic potential, pathogen transmission, and bacterial pathogenesis. It is hypothesised that the differential virulence illustrated by previous *in vivo* experimentation is driven by differences in type III secretion with differences at both the genome level and how much protein is secreted controlling the outcome of infection.

This project aims to characterise the secretomes of well-characterised strains of *S.* Typhimurium and *S.* Choleraesuis to improve upon livestock vaccination strategies and identify novel broad-spectrum anti-virulence drug or vaccine candidates.

1. Compare the published genome sequences of ST4/74 and SCSA50.

2. Characterise the secretomes of the strains under T3SS inducing conditions using quantitative proteomics.

3. Investigate differences in regulation under T3SS inducing conditions.

4. Compare invasiveness, intracellular survival and replication of the strains in relevant porcine epithelial cell types.

Chapter 2 Materials and methods

2.1 Bacteriology

2.1.1 Bacterial strains

The bacterial strains used in this project are listed in Table 2.1. The two primary *Salmonella enterica* strains used in this study were *S. enterica* serovar Typhimurium strain ST4/74 (Rankin and Taylor, 1966) and *S. enterica* serovar Choleraesuis strain SCSA50 (Bolton *et al.*, 1999) which were considered representative of their wider serovar (Bolton *et al.*, 1999; Paulin *et al.*, 2007). In several experiments the gene deletion mutant ST4/74 Δ*prgH* which does not have a functional T3SS-1 was used as a negative control.

All strains were maintained on LB (Luria-Bertani) agar plates, supplemented with antibiotics as needed (Ampicillin (Amp) 100 μg/ml, Chloramphenicol (Cmp) 12.5 μg/ml and Kanamycin (Kan) 50 μg/ml).

To generate glycerol stocks of strains a colony was inoculated into 10 ml of LB broth and cultured overnight. 1 ml was pelleted in a bench top centrifuge and resuspended in 1 ml 50% v/v glycerol in LB broth (1:1, sterilised by autoclaving) and appropriate antibiotics before being stored indefinitely at -80 ˚C.

Table 2.1 | *Salmonella enterica* strains used in this thesis.

Abbreviations – *Salmonella* reference collection B (SARB); American Type Culture Collection (ATCC).

Strain		Resistance	Reference/source
***S. enterica* strains**			
S. Typhimurium	ST4/74		Rankin & Taylor 1966
	ST4/74 Δ*prgH*	Kan^R	Mark Stevens lab
	ST4/74 pHSG422	Amp^R Kan^R Cmp^R	Paulin *et al.* 2007
	SL1344 promoterless-GFP$^+$	Cmp^R	Hautefort *et al.* 2003
	SL1344 *rpsM*-GFP$^+$	Cmp^R	Hautefort *et al.* 2003
	SL1344 *prgH*-GFP$^+$	Cmp^R	Hautefort *et al.* 2003
	SL1344 *ssaG*-GFP$^+$	Cmp^R	Hautefort *et al.* 2003
	TML		Giannella *et al.* 1973
	ST12/75		Watson *et al.* 1995
	Bovine clinical isolate R676		Gragg *et al.* 2013
	Bovine clinical isolate R723		Gragg *et al.* 2013
	Bovine clinical isolate R744		Gragg *et al.* 2013
	Bovine clinical isolate R771		Gragg *et al.* 2013
	Bovine clinical isolate R813		Gragg *et al.* 2013
S. Choleraesuis	SCSA50		Bolton *et al.* 1999
	SCSA50 pHSG422	Amp^R Kan^R Cmp^R	Paulin *et al.* 2007
	SC14/74		Bolton *et al.* 1999
	ATCC® 7001™		ATCC
	ATCC® 12011™		ATCC
	ATCC® 13312™		ATCC
	SARB 4		Boyd *et al.* 1993
	SARB 5		Boyd *et al.* 1993
	SARB 6		Boyd *et al.* 1993
	SARB 7		Boyd *et al.* 1993

Table 2.1 | cont.

Strain		Resistance	Reference/source
***S. enterica* strains (cont.)**			
***S.* Abortusovis**	SAO15/5		Colombo *et al.* 1992
	SAO44		Lantier *et al.* 1983
***S.* Agona**	SARB 1		Boyd *et al.* 1993
***S.* Derby**	SARB 9		Boyd *et al.* 1993
	SARB 10		Boyd *et al.* 1993
***S.* Dublin**	SD3246		Hall & Jones 1976
	SD2229		Baird *et al.* 1985
***S.* Enteritidis**	SARB 16		Boyd *et al.* 1993
	SARB 17		Boyd *et al.* 1993
***S.* Gallinarum**	SG9		Williams 1955
	SARB 21		Boyd *et al.* 1993
***S.* Heidelberg**	SARB 23		Boyd *et al.* 1993
	SARB 24		Boyd *et al.* 1993
***S.* Infantis**	SARB 26		Boyd *et al.* 1993
	SARB 27		Boyd *et al.* 1993
***S.* Montevideo**	SARB 30		Boyd *et al.* 1993
	SARB 31		Boyd *et al.* 1993
***S.* Saintpaul**	SARB 56		Boyd *et al.* 1993
***S.* Typhisuis**	SARB 70		Boyd *et al.* 1993

2.1.2 Induction of *S. enterica* type III secretion system 1

The conditions used to induce secretion associated with T3SS-1 encoded by *Salmonella* pathogenicity island 1 (SPI-1) were adapted from several studies (Davis and Mecsas, 2007; Hébrard *et al.*, 2011; Kröger *et al.*, 2013).

Bacteria were cultured overnight at 30 ˚C, shaking at 180 RPM, in Luria-Bertani broth (LB) before adjustment of the OD_{600} (OD) to 1. Subsequently, the bacteria were sub-cultured 1:20 in a new 50 ml falcon tube in 10ml fresh LB broth at 37 ˚C for 4 hours to induce type III secretion-associated protein secretion. Experimental endpoint viable counts were determined by plating serial dilutions on nutrient agar.

2.1.3 Induction of *S. enterica* type III secretion system 2

The minimal media used to induce T3SS-2 was composed of 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$; 0.5 mM K_2SO_4, 8 µM $MgCl_2$, 1 mM Na_2HPO_4, 100 mM BIS-TRIS HCl, pH 7, 38 mM glycerol and 1% (w/v) casamino acids in distilled water. The media composition and protocol was adapted from three publications (Deiwick *et al.*, 1999; Yu *et al.*, 2010; Niemann *et al.*, 2011). Two bottles of media were prepared and the pH adjusted separately. One was adjusted to pH 5.8 and one to pH 7.2 before sterilisation by autoclaving.

Bacteria were cultured overnight at 37 ˚C, shaking at 180 RPM, and the OD adjusted to 1. 500 µl was pelleted using a bench top centrifuge and the cells resuspended in 500 µl T3SS-2 media (pH 5.8). Centrifugation and resuspension were then repeated a further two times to wash residual LB from the bacteria. The pellets were resuspended in a final 500 µl T3SS-2 media (pH 5.8) which was then mixed with 9.5 ml fresh T3SS-2 media (pH 5.8).

The bacteria were then cultured for 6 hours at 37 ˚C, shaking before centrifugation (3220 RCF, 21 ˚C, 8 minutes) and resuspension in 10 ml fresh T3SS-2 media (pH 7.2). The bacteria were then cultured overnight before

experimental endpoint viable counts were determined by plating serial dilutions on nutrient agar.

2.1.4 Measuring GFP expression by fluorescent GFP+ promoter fusions

To determine the transcription of specific genes under experimental conditions, genetically modified strains of *S.* Typhimurium SL1344 were used. SL1344 is a histidine auxotroph (Hoiseth and Stocker, 1981) with only 8 SNPs differentiating it from ST4/74 (Richardson *et al.*, 2011). The strains were engineered as single-copy GFP gene fusions (Hautefort *et al.*, 2003) with *gfp* fused to the promoter regions of genes of interest such as *prgH* and *ssaG,* two genes which encode components of the T3SS-1 or T3SS-2 needle.

The strains were cultured either under T3SS-1 or T3SS-2 conditions as described above and at specific time points, 200 µl was transferred to a clear flat-bottomed 96-well plate for determination of both optical density and GFP using a Cytation 3 microplate reader (BioTek).

2.1.5 Assessment of bacterial swimming motility

Motility agar was prepared on the day of the experiment in distilled autoclaved water (0.5% w/v peptone, 0.3% w/v yeast extract, 0.3% w/v Difco Bacto-agar) and autoclaved.

After sterilisation, motility plates were prepared in standard petri dishes two hours before experimentation. Within a laminar flow cabinet and using a serological pipette, 25 ml of the hot agar was transferred to a petri dish which was allowed to cool and semi-solidify for 15 minutes.

Bacteria were cultured under T3SS-1 inducing conditions described previously and diluted in fresh LB broth 1:10 motility was assessed.

3 µl was then carefully spotted on the centre of the motility plate without stabbing the agar itself. The plates were kept upright and incubated overnight

at 37 ˚C before the diameter of bacterial swimming measured from the inoculation point. The plates were imaged using the G:BOX image capture system (Syngene).

2.1.6 Growth curves

Bacteria was cultured as previously described, either under T3SS-1 or T3SS-2 inducing conditions. At each hour of interest 200 µl of culture was transferred to a clear flat-bottomed 96-well plate for determination of optical density (measured at wavelength 600 nm) using a Cytation 3 microplate reader (BioTek).

2.2 Molecular biology

2.2.1 Extraction of bacterial and plasmid DNA from cultures and colonies

Bacterial genomic DNA (gDNA) was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions for Gram-negative bacteria. DNA was extracted from 1.5 ml of overnight culture.

Plasmids were extracted from bacteria using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Each time, 1.5 ml of a 5 ml overnight culture was pelleted for plasmid extraction and DNA eluted in 20 µl nuclease free water.

The quality and quantity of extracted gDNA and plasmid DNA was checked by mounting 1 µl on a ND-1000 spectrophotometer (Thermo Fisher Scientific) and the DNA samples were considered pure if there was a single spectral peak detected and the $A_{260/280}$ ratio between 1.6 and 1.9 and the $A_{260/230}$ ratio between 2 and 2.2.

To screen bacterial colonies by Polymerase Chain Reaction (PCR), PCR tubes were first prepared with nuclease free water (50-60% v/v of the PCR reaction depending on the template and primer concentration). Using a 10 µl sterile pipette tip under an open flame, colonies were lightly touched and the

tip resuspended in the water before the tip was removed and the remaining PCR reaction components added.

2.2.2 Transformation of bacteria by electroporation

Electrocompetent cells were always prepared fresh on the day of the transformation. From an overnight culture (37 ˚C, shaking at 180 RPM) 120 µl was diluted in 12 ml of LB broth in an autoclaved glass flask (1:100) which was then cultured (37 ˚C, shaking at 180 RPM) until the OD was between 0.6 and 0.8. The culture was then chilled on ice for 20 minutes and then pelleted by centrifugation (3220 RCF, 4 ˚C, 8 minutes) and carefully resuspended in cold 10% v/v glycerol. The cells were washed by three further centrifugations (four in total) and finally resuspended in 100 µl 10% glycerol. On ice, the plasmid of interest was incubated with 50 µl electrocompetent cells for 30 minutes before the mix was transferred to a pre-chilled electroporation cuvette with a 2 mm gap (Bio-Rad). The electroporation was performed using a Gene Pulser Xcell machine using a single pulse of 25 µF, 1.8 kV and 200 Ω. 1 ml of either LB broth or super optimal catabolite repression (SOC) medium was added immediately and the pulsed cells transferred to a fresh tube for 2 hours recovery shaking at the appropriate temperature. 50 µl of the transformation was plated on LB agar with relevant selective antibiotics. As a negative control, 50 µl of competent cells which were not incubated with the plasmid was plated on LB antibiotic agar in every experiment.

Occasionally, electrocompetent cells were prepared directly from overnight cultures (Choi, Kumar and Schweizer, 2006). At room temperature, 6 ml of overnight culture was pelleted and resuspended in 300 mM sucrose twice before final resuspension in 100 µl 300 mM sucrose. Plasmid was added to 50 µl of cells which were then incubated on ice for 30 minutes prior to transfer to an electroporation cuvette. Electroporation and recovery were performed as described above.

2.2.3 Polymerase chain reaction (PCR) and gel electrophoresis

PCRs were performed in either 12.5 or 25 µl volumes typically composed of 0.5 units of polymerase (either Phusion (New England BioLabs) or GoTaq G2 (Promega)), 1x polymerase buffer, 10 mM deoxynucleotides (dNTPs), nuclease free water (50-60% v/v), 0.5-1 µM oligonucleotide primers, dimethyl sulphoxide (DMSO (3% v/v) and either 0.5-1 µl DNA template (1% v/v) or 1 µl nuclease free water if colony DNA is the template or it was a negative control.

For conventional PCR to check qPCR primers, conditions were constant for all primer pairs. PCR was performed as follows – 98 ˚C for 1 minutes then 35 cycles of 98 ˚C for 10 seconds and 60 ˚C for 30 seconds, and 72 ˚C for 30 seconds before a final extension step at 72 ˚C for 2 minutes.

Primers were designed using the NCBI Primer- BLAST server (Ye *et al.*, 2012), using the ST4/74 and SCSA50 genomes (Richardson *et al.*, 2011). Primers were designed to amplify products between 100 and 200 bp which were 100% conserved between the two strains. Primers used in this thesis are listed in Table 2.2.

Table 2.2 | Primers used in this thesis.

Primer	Sequence (5' – 3')	Description
qPCR primers		
rpoD_FWD	TATGGAGCAAAACCCGCAGT	Amplification of the housekeeping gene
rpoD_REV	AGCAGATCATCGGCATCAGG	
hilA_FWD	GGGCAGATGATACCCGATGG	Amplification of the gene of interest
hilA_REV	AAGAGAGAAGCGGGTTGGTG	
hilC_FWD	CACCTCAGCCTGTGACCATT	
hilC_REV	CCCGGTGGGTTTGATTTCCT	
hilD_FWD	TGACGAACCTGGGATGTTGG	
hilD_REV	CTCTGTGGGTACCGCCATTT	
hilE_FWD	GTGATAAGGGCGCAATGCTG	
hilE_REV	CTTTTCGACGCTAATGCGGG	
ssrB_FWD	TGCTACTGTTTGCAATGCCG	
ssrB REV	CTACCTGGCATCAATGGCCT	
phoP_FWD	GGAGGCCAGACCGCTATTAC	
phoP_REV	AGTGATGTTTCACTGCCGGT	
ompR_FWD	CGGATGGCTGACTAACGCTT	
ompR_REV	GTCGTCAGGCAAACGAACTG	
hha_FWD	CTGAAGAGGGGATCTTGTCGT	
hha_REV	TTTGATGCGTTTACGGCGCT	
sipC_FWD	TTGACGCTATTCTGCCCGTT	
sipC_REV	TGAATGCGTTGTCCGGTAGT	
pipB_FWD	TACACCCGTTGACATCCTCC	
pipB_REV	TCACCACGCGGTATACTGGA	
pipB2_FWD	TCGCGTGTTGTAAATTGGCG	
pipB2_REV	GAGGGGGCAGTGCTGTTTAT	
sifA_FWD	CCGCTTTGTTGTTCTGAGCG	

Table 2.2 | cont.

Primer	Sequence (5' – 3')	Description
qPCR primers (cont.)		
sifA REV	AGACGTTTCAGGCGTTCCTC	Amplification of the gene of interest
sicA_FWD	CTGTGCTGCTCTGTCTCCG	
sicA_REV	GCAAAAGCCAGACAGTGTT	
invB_FWD	AATCCGGATGCACTAAGGCT	
invB_REV	ATGTATGGATCTGGGCGCAA	
srcA_FWD	GCTTGTTAGTAAGATACGGTAACGG	
srcA_REV	AGAGCCGATCGCTTATTAAGACAGT	
flgK_FWD	CAGCGCGAATATGATGCGTT	
flgK_REV	ACTAACGTTTGCAGGCTGGT	

Amplified DNA was separated by agarose gel electrophoresis which was prepared using either at 0.8%, 1% or 1.2% (w/v) UltraPure™ agarose (Invitrogen) and 1X SYBR™ Safe DNA Gel stain (Invitrogen) in tris-acetate-EDTA (TAE). PCR products were mixed 6:1 6X Blue/Orange Loading dye (Promega) and electrophoresis performed at 100 V for 45 minutes. Bands were subsequently visualised using ultraviolet light in the G:BOX image capture system (Syngene).

2.3 General protein methods

2.3.1 Precipitation of bacterial proteins

2.3.1.1 Precipitation of proteins after T3SS-1 induction

After the four-hour induction of T3SS-1 (described in section 2.1.2), secreted proteins and cell-associated proteins were separated.

1 ml of culture was removed and pelleted to represent the bacterial cell-associated proteins once lysed. Pellets were typically frozen at -20 ˚C. Secreted proteins were harvested by centrifugation (3220 RCF, 4 ˚C, 30 minutes) and filter-sterilised with a 0.2 µm low-protein binding filter to exclude any whole-cell bacteria. Proteins were then precipitated using pyrogallol red-molybdate methanol (PRMM) (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1 mM sodium oxalate, 50 mM succinic acid, 20% w/v MeOH, pH 2.0) (Caldwell and Lattemann, 2004). An equal volume of PRMM was added to the supernatants and the pH of the mixture adjusted to 2.8 by concentrated HCl (0.2% v/v) before overnight precipitation at 4 ˚C in darkness.

Purple PRMM-bound secreted proteins were collected by centrifugation (3220 RCF, 4 ˚C, 30 minutes) and removal of the supernatant. Proteins were washed twice in ice-cold 100% acetone before being solubilised in 50 mM ammonium bicarbonate for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) or a peptide digestion buffer prior to mass spectrometry (8 M urea, 0.1% w/v SDS and 50 mM ammonium bicarbonate in molecular grade water).

Cell-associated pellets were thawed and washed by being resuspended in PBS and pelleted be centrifugation for lysis prior to SDS-PAGE separation.

Secreted protein quantification was performed using a Qubit® Protein Assay Kit (Invitrogen) and Qubit fluorometer (Invitrogen).

2.3.1.2 Precipitation of proteins after T3SS-2 induction

After the induction of T3SS-2 (described in the relevant bacteriological methods section 2.1.3), secreted proteins and cell-associated proteins were separated.

The cell-associated proteins were prepared using the same method as under T3SS-1 conditions.

Secreted proteins were isolated by centrifugation (3220 RCF, 4 ˚C, 30 minutes) and filter-sterilised with a 0.2 µm low-protein binding filter to exclude any whole-cell bacteria. Proteins were then enriched and precipitated using StrataClean resin (Agilent) (Otto *et al.*, 2017). The resin was thoroughly vortexed and 50 µl added to the tube containing the supernatant (1:200). The tube was vortexed for 15 seconds and transferred to a 2D rocker for 30 minutes.

Protein bound to resin was collected by centrifugation (3220 RCF, 4 ˚C, 30 minutes) and removal of the supernatant. The pellet was washed with PBS, pelleted and allowed to dry on the bench (6 hours – overnight) before SDS-PAGE separation.

2.3.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE under reducing conditions prior to visualisation by staining or western blotting.

To separate the cell associated proteins, the washed pellets (described above) were lysed by resuspension in 400 µl of Laemmli buffer (100 mM Tris pH 6.8, 4% w/v SDS, 0.2% w/v Bromophenol blue, 20% v/v glycerol and 2% β-mercaptoethanol. For a single well, 1 µg of secreted proteins after T3SS-1

induction was mixed 1:1 with Laemmli buffer. For T3SS-2 secreted samples, the dried resin was resuspended in 50 µl of Laemmli buffer.

The protein samples, either cell-associated or secreted, were boiled at 99 ˚C for 10 minutes and subsequently centrifuged (13,000 RPM, room temperature, 10 minutes).

Gels were either purchased pre-cast 12% Novex™ WedgeWell™ Tris Glycine (Thermo Fisher Scientific) or 3-8% NuPAGE™ Bis-Tris (Thermo Fisher Scientific) protein gels or prepared in the lab. 12% Tris Glycine gels were prepared and constituted a resolving gel and stacking gel. The resolving gel was composed of 2.33 ml 30% Acrylamide/Bis-acrylamide solution (Sigma Aldrich), 1.88 ml 1 M Tris-HCl pH 8.8, 50 µl 10% w/v SDS, 0.75 ml distilled water, 4.17 µl tetramethylethylenediamine (TEMED) and 16.67 µl 10% w/v ammonium persulphate. The stacking gel was prepared with 0.3 ml 30% Acrylamide/Bis-acrylamide solution (Sigma Aldrich), 157.5 µl 1.5 M Tris-HCl pH 6.8, 12.5 µl w/v SDS, 0.78 ml distilled water, 3.13 µl TEMED and 6.25 µl 10% w/v ammonium persulphate.

Prior to samples being loaded, the gels were submersed in 1x Tris Glycine SDS running buffer (Bio-Rad) or 1x NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific) depending on the composition of the gel.

After boiling and centrifugation, samples were loaded either as a specific quantity (T3SS-1 secreted proteins) or volume (10 µl resin bound T3SS-2 induced proteins and 15 µl cell-associated proteins). Either the Prism Ultra (Abcam) ladder (10-245 kDa) or the HiMark (Thermo Fisher Scientific) ladder (30-460 kDa) was used as a pre-stained protein standard. Proteins were separated by electrophoresis at 150 V for 60 minutes.

2.3.3 Coomassie blue and silver staining

After separation by SDS-PAGE, proteins were stained with either Coomassie blue or silver stain for profile visualisation.

Gels were covered in 10-15 ml Coomassie Brilliant Blue G-250 (Bio-Rad) and incubated for 45 minutes on a 2D rocker before submersal in de-stain solution (40% v/v methanol, 10% v/v acetic acid) for 1 hour. The gels were washed with either de-stain or water in 15-minute intervals until protein bands were clearly discernible.

To detect low amounts of protein, silver was employed as a highly sensitive stain which bound to protein present as low as 0.25 ng. The Pierce™ Silver Stain kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Gels were washed in water, fixed (30% v/v ethanol, 20% v/v acetic acid), washed in 10% ethanol and then in water before sensitisation and staining. After staining, gels were washed in water and then developed until bands were detectible by eye. 5% v/v acetic acid stopped the development reaction.

2.3.4 Western blotting

Western blotting was performed after SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad) using the manufacturer's program for mini gels. Transfer (Towbin) buffer consisted of 25 mM Tris, 192 mM glycine and 20% v/v methanol.

All subsequent steps were performed using a 2D rocker. After transfer (success determined by ladder transfer) the membrane was incubated with blocking buffer 5% w/v non-fat milk in PBS 0.1% v/v Tween-20 (PBST) for 1 hour at room-temperature or overnight at 4 ˚C. After blocking the membrane was washed for 15 minutes and then 2 x 5 minutes in fresh PBST. To probe for proteins of interest, monoclonal antibodies were diluted in PBST to a specific concentration. Antibodies used in this thesis are listed in Table 2.3.

Table 2.3 | Antibodies used in this thesis.

Primary antibody			
Protein target	**Species**	**Concentration**	**Source**
SipA	Murine	0.2 µg/ml	In house
SipC	Murine	0.2 µg/ml	In house
SopB	Murine	0.2 µg/ml	In house
SopE2	Murine	0.2 µg/ml	In house
DnaK (ab69617)	Murine	1:10000	Abcam
DnaK (ab80161)	Rabbit	1:5000	Abcam
Secondary antibody			
Protein target	**Species**	**Concentration**	**Source**
Anti-mouse IgG (DyLight 800 4x PEG)	Goat	1:10000	Invitrogen
Anti-rabbit IgG (Dylight 800 4x PEG)	Goat	1:10000	Invitrogen

The membrane was incubated with the primary antibody either for 1 hour at room temperature or overnight at 4 ˚C. After the primary, membranes were

washed again for 15 minutes then 2 x 5 minutes in PBST. The species-specific secondary antibody (Table 2.3) was then diluted in PBST and the membrane incubated for 45 minutes at room temperature. After the secondary antibody was added all incubation and wash steps were performed in darkness. The membrane was washed for 15 minutes then 2 x 5 minutes in PBST before being scanned on an Odyssey imager using ImageStudio software (LI-COR Biosciences).

2.4 Proteomics

2.4.1 Shotgun proteomics

Shotgun proteomics was performed by Dr Dominic Kurian, Proteomics and Metabolomics Facility manager at the Roslin Institute according to facility standard operating procedures using HPLC-MS. Samples were provided to the facility in the previously described peptide digestion buffer.

Under the supervision of Dr Dominic Kurian, in-solution digestion was performed. First the samples were reduced with 10 mM dithiothreitol (DTT) for 1 hour at 36 ˚C. To prevent the reformation of disulphide bonds, iodoacetamide alkylation was performed for 45 minutes prior to overnight 1:20 trypsin digestion at 37 ˚C. Trypsin cuts at the C-terminal of amino acid lysine and arginine residues to digest proteins into peptides.

After tryptic digestion, the digested peptides were cleaned up to remove impurities, urea, trypsin and DTT. The samples were first acidified in 0.1% trifluoreacetic acid (TFA) before C^{18} chromatography. Acidified and polarised peptides bind the long linear C^{18} hydrocarbon which was first activated by the addition of acetonitrile. The samples were bound to the hydrocarbon, washed with TFA and eluted with acetonitrile solution. For mass spectrometry, the acetonitrile was evaporated using a vacuum and samples resuspended in a specific buffer.

Identified peptides were matched to proteins in the predicted proteome of ST4/74 and SCSA50 (extracted from UniProt (ST4/74 UP000008978;

SCSA50 UP000003971) using MASCOT server (Matrix Science). Proteins were considered confident matches with > 2 unique peptides identified.

2.4.2 Label-free quantitative proteomics

Quantitative label-free mass spectrometry was performed by scientists at Gemini Biosciences Ltd in Liverpool, England. Three biological replicates of both ST4/74 and SCSA50 proteins secreted under T3SS-1 inducing conditions were generated and sent by post to the company. After Gemini Biosciences quality control, however, all three replicates of ST4/74 passed but only one SCSA50 replicate was of high enough quality. The following protocol was communicated by Dr Paul Ajuh, the company founder.

Samples were dissolved in Laemmli buffer and electrophoretically separated in a polyacrylamide gel for 20 minutes before in-gel digestion (Schevchenko *et al.,* 2007). Samples were then desalted on C18 Micro spin columns (Harvard Apparatus, USA) as described in Bouchal *et al.,* 2009. Desalted tryptic peptides were dissolved in 30 µl of 5% acetonitrile (CAN), 0.05% TFA and separated on liquid chromatograph Eksigent ekspert™ nanoLC 400 (SCIEX, Dublin, CA, USA). Liquid chromatography was online-connected to a TripleTOF 5600+ mass spectrometer (SCIEX, Toronto, Canada). Samples were pre-concentrated on a cartridge trap column (300 µm i.d. × 5 mm) packed with C18 PepMap™ 100 sorbent with 5 µm particle size (Thermo Scientific, MA, USA) using a mobile phase composed from 0.05% trifluoroacetic acid (TFA) in 2% acetonitrile (CAN). The pre-concentrated peptides were separated on a capillary analytical column (75 µm i.d. × 500 mm) packed with C18 PepMap™ 100 sorbent, 2 µm particle size (Thermo Fisher Scientific, MA, USA). Mobile phase A composed of 0.1% (v/v) formic acid (FA) in water while mobile phase B composed of 0.1% (v/v) FA in CAN. Analytical gradient started from 2% B, the proportion of mobile phase B increased linearly up to 40% B in 120 min, flow was 300 nl/min. The analytes were ionized in nano-electrospray ion source, where temperature and flow of drying gas was set to 150ºC and 12 psi. Voltage at the capillary emitter was 2.65 kV.

SWATH (sequential window acquisition of all theoretical fragment ion spectra mass spectrometry) data acquisition was performed in high sensitivity mode and precursor range set from 400 Da to 1200 Da. It was divided to 67 precursor SWATH windows with 12 Da width and 1 Da overlap. Cycle time was 3.5 seconds. Pooled spectral library samples were measured in information dependent mode (IDA). Precursor range was set from 400 Da up to 1250 Da in MS mode and from 200 Da up to 1600 Da in MS/MS mode. Cycle time was set to 2.3 seconds and during each cycle the top 20 most intensive precursor ions were fragmented. Precursor exclusion time was set to 12 seconds. IDA data were then searched against both the ST4/74 (Uniprot proteome ID UP000008978) and the SCSA50 (Uniprot proteome ID UP000003971) reference databases in ProteinPilot™ 4.5 (AB-SCIEX, Canada). Quantitative data extraction for all identified proteins was performed in PeakView® 1.2.0.3. Quantitative data were extracted for 422 proteins (FDR<1%) using a method with 8 minute extraction window and the data analysed in MarkerView™ 1.2.1.1. SWATH data was normalised using flagellar protein FlgK and changes in protein level across samples determined using a t-test.

2.4.3 Data cleaning

For both proteomics datasets, the data had to be cleaned manually due to several incorrect annotations in the genomes of both strains. Basic local alignment search tool (BLAST) for proteins (Altschul *et al.*, 1990) was used to confirm each annotation.

2.5 Reverse-transcriptase quantitative PCR (RT-qPCR)

2.5.1 Extraction of bacterial RNA

To reduce environmental RNase contamination and degradation, RnaseZap (Sigma Aldrich) was used to spray the bench and pipettes prior to RNA extraction.

RNA was thus extracted from ~3x10^8 bacterial cells during induction of T3SS-1 activity at two time points, first after overnight culture at 30 ˚C (baseline) and secondly after subculture at 37 ˚C (induced). The volume of culture to extract RNA from was first optimised using a series of dilutions (5 ml, 1 ml, 500 µl, 250 µl and 125 µl). RNA extracted from a high volume of culture was more likely to be contaminated with gDNA and salts after Dnase treatment and therefore 125 µl (~3x10^8 CFU) was determined to be optimal.

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions from ST4/74 and SCSA50 bacterial cultures grown overnight at 30 ˚C and from cultures after 37 ˚C subculture (T3SS-1 conditions). 125 µl of culture was pelleted at room temperature (13,000 RPM, 10 minutes) and resuspended in 1 ml of TRIzol. 200 µl of chloroform was then added, the tubes vortexed and incubated for 3 minutes. The aqueous phase was then isolated by centrifugation (12,000 G, 4 ˚C, 15 minutes) and mixed in a fresh tube with 500 µl of isopropanol before incubation for 10 minutes. The samples were then centrifuged again (12,000 G, 4 ˚C, 10 minutes), supernatant removed and 1 ml of 75% ethanol (in diethyl pyrocarbonate treated molecular grade water (DEPC water)). The samples were vortexed and centrifuged (7,500 G, 4 ˚C, 5 minutes) before the pellets were air-dried. Finally, 20 µl of DEPC water was added to the air-dried pellets which was then incubated at 55 ˚C for 5 minutes.

Using the ND-1000 spectrophotometer, the quality and quantity of RNA was measured before treatment with Rnase-free Dnase I (Promega) according to the manufacturer's instructions. Briefly, 1 µg of RNA in DEPC water was combined with 1X Dnase 10X reaction buffer and 1 unit of Dnase for 30 minutes at 37 ˚C. 1 µl of Dnase stop solution was added to terminate the reaction, which was then incubated at 65 ˚C for 10 minutes to inactivate the enzymes.

After Dnase treatment the quantity of RNA was again checked using the Nanodrop (ND)-1000 spectrophotometer and the concentration adjusted to

100 ng/µl. 500 ng aliquots were then stored at -20 ˚C prior to cDNA synthesis.

2.5.2 Synthesis of complementary DNA (cDNA)

First strand complementary DNA (cDNA) synthesis using random primers was then performed on 500 ng total RNA using AffinityScript Multi Temp cDNA kit as per the manufacturer's instructions (Agilent Technologies).

All incubation steps unless specified were performed in a PCR machine. 500 ng (5 µl) aliquots of RNA were thawed on ice and combined with 3 µl random primers (0.1 µg/µl) and 7.7 µl Rnase-free water in a sterile PCR tube. The mix was incubated at 65 ˚C for 5 minutes. The tubes were removed from the machine and allowed to cool at room temperature to allow the primers to anneal for 10 minutes. After 10 minutes the following reagents were added – 2 µl reverse transcriptase buffer (1X), 0.8 µl dNTPs (25 mM each dNTP), 0.5 µl Rnase ribonuclease inhibitor (20 units) and 1 µl reverse transcriptase. The tubes were then returned to the PCR machine and incubated at 25 ˚C for 10 minutes then at 55 ˚C for 60 minutes. Finally, synthesis was terminated by incubation at 70 ˚C for 15 minutes.

As a control for gDNA contamination, cDNA synthesis was also performed on RNA without reverse transcriptase. These samples would be used for conventional PCR and qPCR to check for contamination.

2.5.3 Quantitative PCR

Quantitative PCR (qPCR) reactions were performed on three biological replicates and a plate always included three technical replicates of each condition or gene tested for each strain, two negative water controls and three no-template contamination control wells.

Gene-specific oligonucleotides for both *S.* Typhimurium and *S.* Choleraesuis were designed using NCBI Primer-BLAST in identical gene regions (Table 2.2) and were first tested in conventional PCR using gDNA to ensure product specificity. PCR conditions are also described previously.

qPCR reactions were performed in 10 µl reaction volumes (5 µl PerfeCTa SYBR® Green SuperMix low ROX® (Quanta Biosciences), 1 µl forward and reverse primers at optimised concentrations, 1 µl diluted cDNA, and 3 µl Rnase-free water) using the Mx3000P PCR machine (Stratagene) (95˚C 3 min followed by 40 cycles of 95˚C for 10 s and 60˚C for 30 s).

The MxPro software (Agilent Technologies) was used to analyse the data. The reference dye ROX within the qPCR master mix was used to normalise the fluorescent signal, which was automatically computed by the software.

Dissociation curves were included in every experiment to ensure a single product was generated and to determine optimal primer concentration. 100 nM, 200 nM and 300 nM concentrations were tested and the lowest concentration selected which still amplified a single, strong product. To calculate the efficiency of amplification for each product and determine what concentration of cDNA to use, standard curves were first generated using 2-fold cDNA dilutions for each strain at each condition.

The optimised primer and cDNA concentrations for each gene are listed in Table 2.4.

Table 2.4 | Optimised qPCR cDNA and primer concentrations

Gene	cDNA concentration	Primer concentration (nM)
rpoD	1:50	200
hilA	1:50	200
hilC	1:50	200
hilD	1:50	200
hilE	1:2	300
ssrB	1:50	300
phoP	1:50	300
ompR	1:10	300
hha	1:50	300
sifA	1:50	300
pipB	1:50	200
pipB2	1:50	100
sipC	1:50	300
sicA	1:50	200
invB	1:50	200
srcA	1:50	200
flgK	1:50	300

2.5.4 Data analysis

For experimental qPCR, Ct values were extracted from the MxPro software and compared to the standard curves generated using dilutions of the same cDNA. The amplification and dissociation curve of each replicate was always checked to ensure reaction specificity.

Expression of genes of interest (GOI) was normalised to *rpoD* using the Pfaffl method (Pfaffl, 2001), with samples extracted from overnight cultures used as baseline for expression. The equation was as follows:

$$\frac{(GOI\ Rsq.^{\Delta Ct\ GOI\ (Baseline\ Ct - Induced\ Ct)})}{(rpoD\ Rsq.^{\Delta Ct\ rpoD\ (Baseline\ Ct - Induced\ Ct)})}$$

The Pfaffl analysis takes the Rsq. Efficiency value generated by the standard curve into consideration which is an important feature.

Secondary analysis was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), studying transcription of only the induced cultures. The equation was as follows:

$$2^{-\Delta\Delta Ct(GOI\ \Delta Ct - rpoD\ \Delta Ct)}$$

2.6 *In vitro* cell infections

2.6.1 Cell line maintenance

The IPEC-J2 cell line, an immortalised cell line derived from the jejunum of a neonatal piglet, was cultured in DMEM/F12 (Thermo Fisher Scientific), a 1:1 mixture of Dulbecco's modified essential medium (DMEM) and Ham's F-12 mediums while the PK-15 cell line, a porcine kidney cell line, was maintained in DMEM (Sigma Aldrich). Both mediums were supplemented with 10% v/v heat-inactivated foetal calf serum (Thermo Fisher Scientific) and 5% v/v penicillin-streptomycin solution (Thermo Fisher Scientific).

During passage, cells were washed twice with phosphate buffered saline (PBS) and detached with 1x Trypsin-EDTA (Gibco) which was incubated for

5 minutes at 37 ˚C. The cells were dislodged and an equal volume of the appropriate media (DMEM/F12 or DMEM) mixed with the Trypsin. Cells were routinely diluted 1:10 and cultured in new flasks until confluent.

Before infection, 1 x 10^5 cells/well were seeded in 24 well plates and were infected once confluent.

For long term storage of cell lines, cells were counted and suspended in DMEM supplemented with 10% v/v FBS and 10% v/v DMSO at a final concentration of 1 x 10^6 cells/ml. Cryovials were kept at either -80 ˚C for short periods of time or -150 ˚C for long term storage.

2.6.2 Gentamicin protection assay

Gentamicin protection assays were routinely performed to assess the invasiveness of bacteria.

Bacteria were cultured overnight at 25 ˚C before adjustment of OD600 to 0.5. After overnight culture the cultures were estimated to contain ~1x10^9 CFU/ml (determined by prior serial dilutions at the same time point). Serial dilutions were performed and plated on LB agar to retrospectively confirm CFU/ml and calculate the multiplicity of infection (MOI).

The media was removed from confluent monolayers of IPEC-J2 or PK-15 cells which were then washed with PBS before infection.

An estimated ~4x10^6 bacterial cells (MOI 20) were mixed with 500 µl of cell culture media for a single well (inoculum). Per strain, infections were performed in duplicate or triplicate depending on the experiment. The inoculum was added slowly down the side of each well and uninfected control wells included. To bring bacteria into contact with the monolayer, the plate was centrifuged (200 G, 21 ˚C, 5 minutes).

The plate was then incubated for 30 minutes at 37 ˚C before the supernatant overlaying the cells was removed and replaced with fresh culture media

supplemented with 5 mg/ml gentamicin to kill extracellular bacteria. The plate was then returned to 37 ˚C for a further 30 minutes.

The supernatant containing gentamicin was then removed and 200 µl 0.1% v/v Triton-X-100 added. After 15 minutes incubation at room temperature, the cells were scraped from the plastic using a pipette tip and transferred to a microcentrifuge tube. Serial dilutions were made in PBS using a 96 well plate and a multi-channel pipette. Neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated for each sample on an LB agar plate by spotting 4-6 10 µl drops of each dilution (Thomas *et al.*, 2015). 100 µl of the lysed uninfected cells was also plated to ensure no cross-contamination occurred.

Plates were incubated at 37 ˚C overnight and bacterial colonies counted to calculate CFU/well. Invasion was then quantified as a % of the total bacteria within the inoculum.

2.6.3 Intracellular survival assay

Gentamicin protection was performed as described in section 2.6.2 and bacteria lysed at 1 hour, 6 hours and 24 hours after initial infection.

The supernatant overlaying the cultures at 6 or 24 hours was collected and immediately stored at -20 ˚C for future cytotoxicity tests.

2.6.4 Plasmid partitioning

Plasmid partitioning in culture was performed using ST4/74 and SCSA50 strains transformed with pHSG422. Strains were cultured overnight either at 25 ˚C, shaking with antibiotics or at 30 ˚C, static with antibiotics.

Bacteria under both culture conditions were adjusted to an OD of 1 before subculture either 1:20 (25 ˚C cultures) or 1:10 (30 ˚C cultures). Subculture was performed without antibiotics and at 37 ˚C, shaking for 4 hours. Serial dilutions were made every hour and plated both on LB agar with ampicillin, chloramphenicol and kanamycin (pHSG422 positive bacteria) and LB agar

alone (total bacteria) to determine whether pHSG422 was titrated out of the population at 37 ˚C with no antibiotic pressure.

Culture overnight at 30 ˚C statically and 1:10 subculture (as described above) for two hours was determined to lead to optimal partitioning in culture. The conditions were then used to prime the strains for invasion and intracellular survival.

Gentamicin protection assays were performed as section 2.6.2 but the cells were lysed only at hour 6 and hour 24 after infection to enumerate intracellular bacteria as described above.

LB plates (with or without antibiotics) were incubated at 30 ˚C overnight.

After quantifying CFU/well, the % plasmid bearing bacteria (isolated on LB agar with antibiotics) was calculated from the total bacteria (isolated on LB agar alone).

2.6.5 Chloroquine resistance assay

To perform a chloroquine resistance assay, a gentamicin protection assay was first performed as described in section 2.6.2 with either 4 or 6 wells/strain.

At 6.5 hours post infection, the media overlaying the cells was removed and replaced with fresh culture media containing either gentamicin alone or gentamicin and 50 μM chloroquine. The concentration of chloroquine was determined not to be cytotoxic to uninfected cells prior to experimentation. The cells were then incubated for a further 1.5 hours (8 hours in total).

At the eighth hour the cells were lysed and intracellular bacteria enumerated as previously described.

2.6.6 Cellular cytotoxicity assessment

Release of lactate dehydrogenase (LDH) by host cells at later time points during experimentation (6, 8, 24 hours post infection) was measured using

the CytoTox 96 Non-Radioactive cytotoxicity assay kit (Promega), following the manufacturer's instructions.

One technical replicate supernatant from each above experiment which were frozen (intracellular survival, plasmid partitioning and chloroquine resistance) were thawed at room temperature. 50 µl was then transferred to a 96 well plate in three technical replicates. 50 µl of the kit substrate mix was added to the wells and the plate incubated at room temperature for 30 minutes in darkness. 50 µl of stop solution was then added to each well and the absorbance at 490 nm measured using Cytation 3 microplate reader (BioTek).

To produce the maximum LDH control, uninfected confluent monolayers of each cell type was lysed for LDH assessment. Untreated culture media was also assessed as a blank.

$$100 \times (Experimental - blank)/(Maximum - blank)$$

2.7 Identification of genes and proteins in *S. enterica* genomes

The presence of each genes or proteins were assayed using BLAST, either BLASTn or tBLASTn (Altschul *et al.*, 1990).

To predict hypothetical candidate T3SS effector proteins, amino acid files were downloaded from UniProt, using the NCBI accession number and entered into www.effectors.org (EffectiveDB). Proteins were identified using UniProt ID mapping before proteins of potential interest were identified. Those of which were uncharacterised or putative and had chaperone binding domains within or outwith the expected region were entered into ELM (http://elm.eu.org/) to search for eukaryotic linear motifs present in the sequences.

The accession numbers used in this thesis are listed in Table 2.5.

Table 2.5 | Genome accession numbers accessed on NCBI

Serovar	Strain	NCBI accession number
Typhimurium	ST4/74	CP002487.1
	LT2	NC_003197.2
	14028S	NC_016856.1
	U288	NC_021151.1
	798	CP003386.1
Choleraesuis	SCSA50	CM001062.1
	SC-B67	NC_006905.1
	C500	CP007639.1
	ATCC 10708	CP012344.2
	NCTC5735	NZ_UGXX01000001.1
Derby	NCTC5722	UGYH01000002.1
	201506934	QKVZ01000011.1
Heidelberg	CFSAN067218	CP028311.1
	SARA35	CP019176.1
Infantis	SARB27	CM001274.1
	FARPER-219	CP038507.1
Saintpaul	SARA23	ABAM02000001.1
	SARA26	CP017727.1
Enteritidis	EC20110353	CP007251.1
	ATCC 9184	NZ_CP019035.1
Dublin	SD3246	NZ_CM001151.1
	2229	LK931502.1
Gallinarum	SG9	NZ_CM001153.1
	287/91	NC_011274.1
Typhi	Ty2	AE014613.1
	CT18	AL513382.1
Paratyphi C	RKS4594	CP000857.1

2.8 Software

GraphPad Prism 9.0 was used to perform all statistics, detailed in the specific results sections. The main statistic applied was the student's t test and *p* values less than 0.05 were considered significant. BLAST Ring Image Generator (BRIG) (Alikhan *et al.*, 2011) was used to visualise the shared sequence identity between the ST4/74 and SCSA50 genomes. Open reading frames were visualised in SnapGene viewer. Heat maps were generated using ggplot2 in Rstudio (Ginestet, 2011).

Chapter 3 The secretome of *S.* Typhimurium ST4/74 and *S.* Choleraesuis SCSA50

3.1 Introduction

Prokaryotic secretion of proteins is a common virulence strategy of many Gram-negative bacteria such as *Escherichia coli, Shigella spp., and Salmonella enterica.* Of the ten identified secretion systems, the role of type III secretion systems (T3SS) and their substrate effector proteins in *Salmonella* virulence have been well-characterised. *S. enterica* encodes two T3SSs which both have pivotal roles in animal models of infection (Carnell *et al.*, 2007; Chaudhuri *et al.*, 2013; Vohra *et al.*, 2019) – the *Salmonella* Pathogenicity Island 1 (SPI-1) Inv-Mxi-Spa family T3SS (T3SS-1) and the SPI-2 Ssa-Esc family T3SS (T3SS-2). Both systems are encoded on large GC-rich chromosomal islands acquired by horizontal transfer and were discovered in 1992 and 1995, respectively (Lee *et al.,* 1992; Hensel *et al.,* 1995).

First discovered in the plague-pathogen *Yersinia pestis* (Zink *et al.*, 1980), T3SSs are 60 nm long nanomachines that extrude from the bacterial cell surface and facilitate the channelled and hierarchical secretion of substrate effector proteins (Salmond and Reeves, 1993; Kubori *et al.*, 1998). *S. enterica* uses its T3SSs to exert effects upon the host by inhibition, manipulation, or stimulation of host-cell processes (Galan and Collmer, 1999; Coburn *et al.*, 2006; Galan and Wolf-watz, 2006).

In cellular models of infection, T3SS-1 is active in the extracellular environment and facilitates the invasion of epithelial cells via cytoskeletal remodelling (Kaniga *et al.*, 1995; Zhou, 1999; Zhou *et al.*, 2001; Patel and Galán, 2006; Chang *et al.*, 2007; Myeni and Zhou, 2010). Subsequent secretion of T3SS-2 effectors promote the bacterial intracellular niche within the acidic *Salmonella*-containing vacuole (SCV) which is anchored by an effector protein-potentiated network of filaments and microtubules (Brumell *et*

al., 2002; Miao *et al.*, 2003; Salcedo and Holden, 2003; Abrahams and Hensel, 2006; Domingues *et al.*, 2014).

While T3SSs, including both T3SS-1 and -2, are highly conserved within the Enterobacteriaceae, T3SSs are distinct in their stimuli and substrate effector proteins (Rosqvist *et al.*, 1995; El qaidi *et al.*, 2017). For example, whilst *S. enterica* readily secretes T3SS-1 invasion-associated effectors into culture supernatants, the Ysc *Yersinia spp*. T3SS is induced by serum albumin (Lee *et al.*, 2001) and calcium starvation at 37 ºC (Michiels *et al.*, 1990) and the *Shigella* spp. T3SS, which is stimulated by host cell contact (Veenendaal *et al.*, 2007) can be induced in culture by the addition of the organic compound Congo red (Parsot *et al.*, 1995).

The two *S. enterica* serovars of greatest interest in this thesis are Typhimurium and Choleraesuis. Both reside within the same Clade A (den Bakker *et al.*, 2011) or are split between two separate groups based on ancestral descent – *S.* Choleraesuis in Group 1 and *S.* Typhimurium in Group 2 (Didelot *et al.*, 2011). Both serovars are natural pathogens of swine (Bolton *et al.*, 1999; Paulin *et al.*, 2007; Campos *et al.*, 2019) and humans (Hsueh *et al.*, 2004; Jones *et al.*, 2008; Havelaar *et al.*, 2015; Ferstl *et al.*, 2017) but differ by disease presentation and outcome (summarised in Figure 1.2).

S. Typhimurium is a host-generalist which causes enteritis in many natural hosts while *S.* Choleraesuis is host-adapted with two natural hosts (swine and humans). With few exceptions, host-adapted *Salmonella* serovars like *S.* Choleraesuis are able to escape the competitive, oxygen-starved intestinal niche and migrate to systemic sites while causing little intestinal inflammation and diarrhoea (Smith and Jones, 1967).

Whilst *S.* Typhimurium has been subject to overwhelming investigation, few studies have explored the molecular mechanisms underlying *S.* Choleraesuis virulence (Sansone *et al.*, 2002; Lichtensteiger and Vimr, 2003; Paulin *et al.*,

2007). Previous research, however, has identified that *S.* Choleraesuis replicates slower and induces less fluid secretion and inflammation in the porcine intestine while secreting less *Salmonella* invasion protein C (SipC) than *S.* Typhimurium (Paulin *et al.*, 2007). SipC is an essential component of the T3SS-1 translocon which localises to host cell membranes (Scherer, Cooper and Miller, 2000; Lara-Tejero and Galán, 2009), ensures translocation of T3SS-1 substrate effector proteins and drives bacterial invasion (Hayward and Koronakis, 1999; McGhie *et al.*, 2001).

This suggested that the repertoire, amount of protein, or shared sequence identity of T3SS substrates could explain the differential *in vivo* virulence showcased by *S.* Typhimurium and *S.* Choleraesuis (Paulin *et al.*, 2007).

Evidence has mounted for the role of secreted effector proteins in the human-restriction of *S.* Typhi, the causative agent of typhoid fever (Spanò *et al.*, 2011; Spanò and Galán, 2012; Nuccio and Bäumler, 2014; Métris *et al.*, 2017), the swine-restriction of *S.* Derby sequence type 40 (ST40) (Tambassi *et al.*, 2020) and the systemic disease caused by sub-Saharan African *S.* Typhimurium ST313 (Carden *et al.*, 2017).

The absence of the genes encoding functional T3SS effectors GtgE and SopD2 in the genome of *S.* Typhi results in decreased bacterial survival in murine epithelial cells and macrophages because the bacteria cannot control the maturation of the SCV (Spanò *et al.*, 2011; Spano *et al.*, 2016) while in human cells *S.* Typhi can survive by an unknown human-specific mechanism (Baldassarre *et al.*, 2021).

Whilst well-characterised ST313 strain D23580 encodes *gtgE*, its genome contains a degraded and therefore non-functional T3SS-2 effector *sseI,* also encoded on the same Gifsy-2 prophage island as *gtgE* (Carden *et al.*, 2017). The absence of the effector SseI contributes to the increased dissemination of D23580 systemically to the mesenteric lymph nodes in a mouse model of infection (Carden *et al.*, 2017).

S. enterica serovar Derby is one of the most common serovar isolated from swine in the European Union (EFSA, 2019) and the swine-adaptation of *S.* Derby ST40 strain ER278 is caused by a single amino acid substitution in the sequence of T3SS-1 transcriptional activator *hilD* (Tambassi *et al.*, 2020).

These studies provide striking mechanistic evidence that the *S. enterica* T3SSs significantly influence the adaptation of serovars to a specific host or environmental niche.

Several genomic and transcriptomic inter-serovar comparisons have illuminated serovar-specific gene carriage and expression profiles (McClelland *et al.*, 2004; Porwollik *et al.*, 2005; Langridge *et al.*, 2015; Johnson *et al.*, 2018a; Martinez-Sanguiné *et al.*, 2020), but there has been only one inter-serovar secretome comparison published (Elhadad *et al.*, 2016). Using a quantitative proteomic approach it was discovered that human-restricted *S.* Paratyphi A secretes less T3SS-1 effectors than *S.* Typhimurium (Elhadad *et al.*, 2016).

S. Typhimurium alone has been subject to extensive proteomic investigation of both the cell-associated proteome and the secretome, which has arguably led to its complete characterisation within *in vitro* conditions mimicking both the SCV (Auweter *et al.*, 2011; Niemann *et al.*, 2011; Sherry *et al.*, 2011; Brown *et al.*, 2012) and the intestinal lumen (Cheng *et al.*, 2017).

The secretion profiles of *S.* Choleraesuis and other livestock-adapted serovars, however, have had little attention. For example, a single study published in 2017 profiled the secreted outer membrane vesicle (OMV) proteome of *S.* Choleraesuis and detected the presence of several T3SS effectors including SipA, SipB, SipC, and SopB in OMVs (Liu, 2017). Profiling the absolute secretome of understudied serovars such as *S.* Choleraesuis could lead to the discovery of serovar-specific virulence factors associated with differential virulence.

Secreted effectors are immunogenic bacterial proteins and have previously been shown to offer protection against *S. enterica* previously as subunit vaccines (Carnell *et al.*, 2007; Jneid *et al.*, 2020). Identifying both shared and novel proteins secreted by serovars could lead to new targets for inclusion in cross-protective vaccine design or broad-spectrum anti-virulence drugs. There are currently no commercial vaccines that effectively control *S. enterica* intestinal colonisation and dissemination in pigs, denoting the need for a greater understanding of the pathogenesis of livestock-adapted serovars.

Moreover, understanding the molecular mechanisms that differ in host-generalist *S.* Typhimurium strain ST4/74 and host-adapted *S.* Choleraesuis SCSA50, which is hypothesised to be correlated with protein secretion, will lead to a greater understanding of zoonotic potential, pathogen transmission, and bacterial pathogenesis.

3.2 Chapter objectives

1. To survey the shared identity at the nucleotide and amino acid level between ST4/74 and SCSA50.
2. Use machine learning tools to predict novel type III secreted effector proteins in the ST4/74 and SCSA50 published genomes.
3. Investigate the *in vitro* conditions to induce T3SS-1 and T3SS-2.
4. Use shotgun proteomics to test detection of secreted effector proteins in the ST4/74 and SCSA50 secretomes.
5. Quantify differences in protein secretion using a label-free proteomics approach.

3.3 Results and discussion

3.3.1 Shared identity between ST4/74 and SCSA50

Genomic degradation by gene deletion or pseudogene formation has been linked to pathogen adaptation to specific hosts and environmental niches and dispensable genes in preferred hosts have been lost as the pathogen

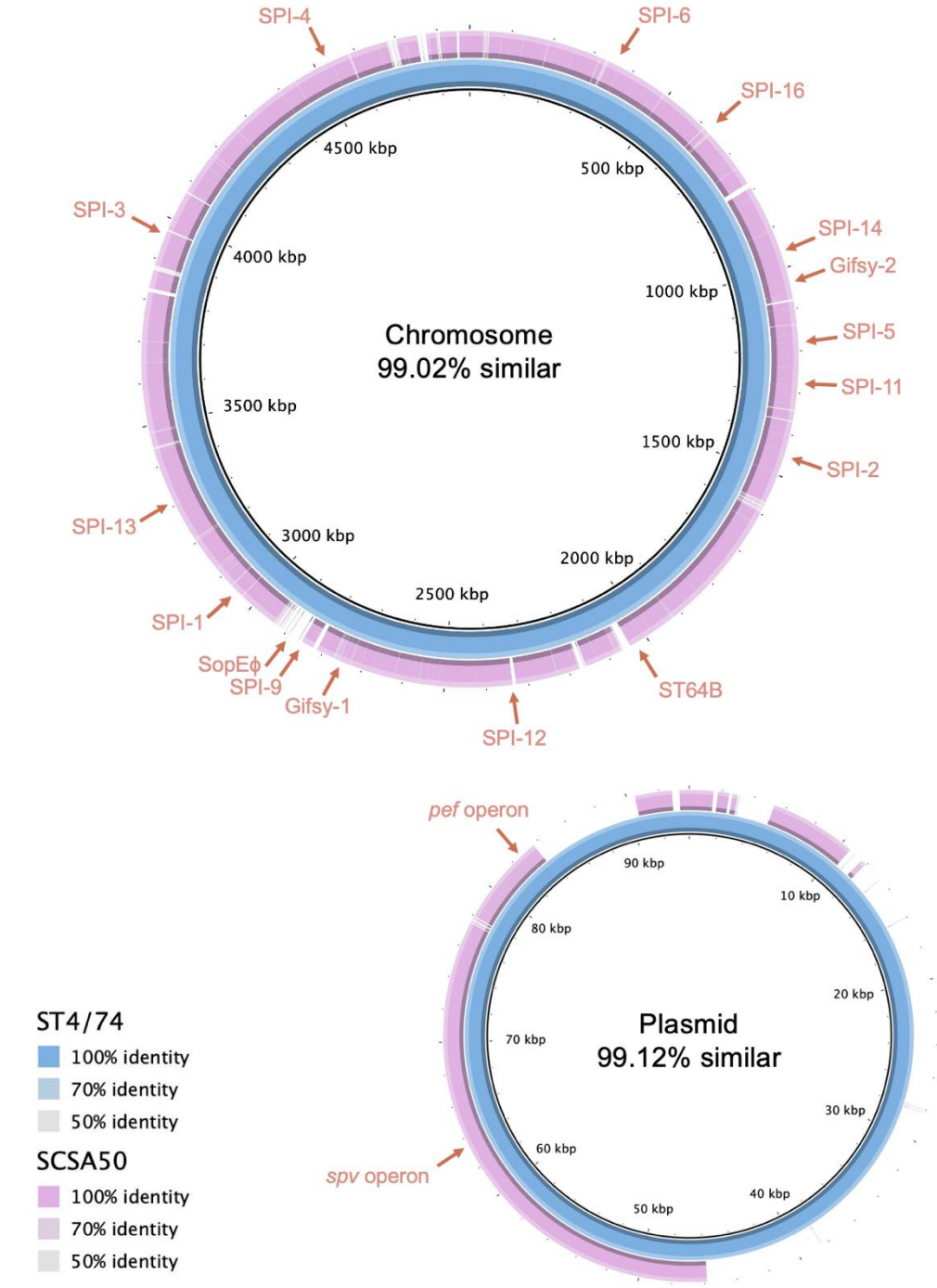
emerges and undergoes host-adaptation (Sakharkar and Chow, 2005; Nuccio and Bäumler, 2014; Langridge *et al.*, 2015).

S. Typhi is a classic example of interlinked genome fragmentation and systemic invasion (Trombert *et al.*, 2011; Spanò and Galán, 2012; Valenzuela *et al.*, 2015), whilst host-adaptation of non-typhoidal *Salmonella* serovars is more complex. Host-adapted pathogens are theorised to have emerged recently from a host-generalist ancestor by accumulation of gene deletions or pseudogenes (Kingsley and Bäumler, 2000; Rakov *et al.*, 2019).

The dynamic nature of bacterial genomes enables *S. enterica* serovars to carry a unique pattern of accessory genes in addition to the core genes shared across the subspecies (Groisman and Ochman, 1996). The possession of major virulence factors T3SS-1 and T3SS-2 on SPI-1 and SPI-2, respectively, are stable and conserved in *S. enterica* but other SPIs and bacteriophage islands can vary between serovars (Fierer and Guiney, 2001).

Pivotally, *Salmonella* divergence from *E. coli* 100 million years ago was defined by acquisition of SPI-1 (Mills *et al.*, 1995). To compare the published genomes and virulence plasmids of ST4/74 and SCSA50 (Richardson *et al.*, 2011), Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) and BLAST Ring Image Generator (BRIG) were employed for sequence alignment visualisation (Alikhan *et al.*, 2011) (Figure 3.1).

A


SPI-4
SPI-6
SPI-16
SPI-14
Gifsy-2
SPI-5
SPI-11
SPI-2
ST64B
SPI-12
Gifsy-1
SPI-9
SopEϕ
SPI-1
SPI-13
SPI-3
500 kbp
1000 kbp
1500 kbp
2000 kbp
2500 kbp
3000 kbp
3500 kbp
4000 kbp
4500 kbp
Chromosome
99.02% similar
pef operon
spv operon
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
80 kbp
90 kbp
Plasmid
99.12% similar
ST4/74
100% identity
70% identity
50% identity
SCSA50
100% identity
70% identity
50% identity

B

Present in SCSA50			Absent in SCSA50
> 99% shared identity	**90-95% shared identity**	**< 90% shared identity**	
SPI-1	SPI-3	SPI-16	SopEϕ
SPI-2	SPI-4		
SPI-5	SPI-6		
SPI-13	SPI-9		
SPI-14	SPI-11		
Gifsy-2	SPI-12		
	Gifsy-1		
	ST64B		

Figure 3.1 | Shared nucleotide similarity between ST4/74 and SCSA50.

(3) Whole genome alignment of ST4/74 (blue inner ring) and SCSA50 (lilac outer ring) nucleotide sequences by BLAST Ring Image Generator (BRIG) (Alikhan *et al*., 2011).

- Location of *Salmonella* Pathogenicity Islands (SPIs) and bacteriophage islands indicated on each plot.
- The alignment of the major *Salmonella* virulence plasmid highlighted the significant difference in plasmid size between ST4/74 (blue inner ring) and SCSA50 (pink outer ring).

(B) BLAST alignment of SPIs and bacteriophage islands reveals ST4/74 and SCSA50 share > 95% nucleotide identity with 14/16 islands. The only island absent in the genome of SCSA50 was the SopEΦ bacteriophage.

Comparison of the genome sequences of ST4/74 and SCSA50 illustrated the high degree of similarity between the strains – the bacterial chromosomes and major virulence plasmids (defined as the plasmid which encodes the *spv* genes) both share 99% identity. There were, however, striking differences. The genome of SCSA50 is ~138 kb smaller than ST4/74 (4,740 kb versus 4,878 kb) which could be an indication of gene loss through evolution of host-adaptation.

The SopEΦ bacteriophage is missing from most host-adapted *Salmonella* serovars (Hardt *et al.*, 1998; Mirold *et al.*, 1999), including SCSA50 (Figure 3.1). The prophage encodes T3SS-1 effector *sopE*, a guanine-nucleotide exchange factor (GEF) that promotes bacterial cell invasion and inflammation (Mirold *et al.*, 1999). The absence or disruption of virulence factors such as *sopE* is often used to differentiate between gastrointestinal host-generalists and systemic host-adapted serovars by machine learning (Wheeler *et al.*, 2018).

Whilst loss of virulence factors such as SopE is important, redundancy within repertoires of effector proteins is predicted (Dean and Kenny, 2009) with effector SopE2 which SCSA50 encodes sharing function and homology with SopE (Bakshi *et al.*, 2000). Whilst SopE and SopE2 share functions as GEFs, the proteins activate different Rho-GTPases. Both interact with Cdc42 but only SopE has been found to interact directly with RalA, Rac1 and RhoG (Hardt *et al.*, 1998; Criss *et al.*, 2001; Friebel *et al.*, 2001; Patel and Galán, 2006). Previously published data, for example, demonstrated that RalA activation is important for *Salmonella* cellular invasion (Nichols and Casanova, 2010) and that *sopE* has a role in *S.* Typhimurium enteritis in calves (Zhang *et al.*, 2002). The absence of the 32 kb *sopE* prophage island partially explains the smaller SCSA50 genome (Mirold *et al.*, 1999) but more experimentation is required to explore the impact of the absence of *sopE* on SCSA50.

Another significant difference was the size of the virulence plasmid, which is carried by multiple serovars including Typhimurium, Choleraesuis, Gallinarum, and Enteritidis (Guiney *et al.*, 1995; Fierer and Guiney, 2001). The ST4/74 plasmid was 90 kb whilst SCSA50 carried a plasmid of only 40 kb (Chu *et al.*, 1999), but notably both encoded the *Salmonella* plasmid virulence (*spv*) and plasmid-encoded fimbriae (*pef*) operons. The 8 kb *spv* operon is a critical component of *Salmonella* dissemination and systemic virulence (Gulig and Doyle, 1993; Guiney *et al.*, 1994) while the *pef* operon contains genes encoding fimbriae important in intestinal colonisation of mice (Bäumler *et al.*, 1996). Despite these differences, however, it did not appear that massive genome degradation separates the chromosomal genomes of ST4/74 and SCSA50 as it separates *S.* Typhimurium and human-restricted *S.* Typhi (McClelland *et al.*, 2004).

BLAST alignment of *Salmonella* pathogenicity and lysogenic phage islands (Figure 3.1B) confirmed that all pathogenicity islands and phage islands, with the exception of SPI-16, had more than 96% nucleotide conservation.

The 4.5 kb long SPI-16 was identified in 2006 and is predicted to be an integrated phage island (Vernikos and Parkhill, 2006). It encodes two glucose translocases, *gtrA* and *gtrB*, and an inner membrane protein *gtrC*, and is key to the persistence of *S.* Typhimurium *in vivo* by O-antigen glucosylation which is strongly potentiated by *gtrC* (Bogomolnaya *et al.*, 2008). The inner membrane protein, designated STM474_0576 in ST4/74, shares only 18% amino acid identity with SCA50_0636. Low sequence identity between GtrC protein families is well documented across *S. enterica* (Davies *et al.*, 2013). Determining whether SPI-16 conservation and glucosylation affects the virulence of ST4/74 and SCSA50 *in vivo* is worthy of future research.

Since the conservation of SPI-1 and SPI-2 (Figure 3.1B) did not explain the differential virulence showcased by ST4/74 and SCSA50, it was hypothesised that the predicted amino acid sequences of T3SS effector proteins could differentiate between the strains. It was additionally theorised

that lack of effector protein SipC sequence identity between ST4/74 and SCSA50 would explain western blotting data previously published (Paulin *et al.*, 2007). Furthermore, in addition to absence of virulence factors, allelic variation is an important factor in virulence factor regulation and host-specificity (Trombert *et al.*, 2011; Valenzuela *et al.*, 2015; Yue *et al.*, 2015; Carden *et al.*, 2017; Johnson *et al.*, 2017; Rakov *et al.*, 2019).

The presence of genes encoding known effector proteins were reviewed using tBLASTn (Altschul *et al.*, 1990), and the predicted translation products were aligned using Clustal Omega (Sievers *et al.*, 2014) to calculate % shared identity between *S.* Typhimurium and *S.* Choleraesuis (Table 3.1).

Table 3.1 | The repertoire of encoded T3SS effector proteins

The genomes of *Salmonella* Typhimurium ST4/74 and *Salmonella* Choleraesuis SCSA50 were interrogated using tBLASTn and Clustal Omega.

Effector	ST4/74	SCSA50	
	Presence	Presence	% Identity
T3SS-1 effector proteins			
SipA	Yes	Yes	99.60%
SipB	Yes	Yes	99.80%
SipC	Yes	Yes	100%
SipD	Yes	Yes	99.42%
SopA	Yes	Yes	59.90%
SopB	Yes	Yes	96.90%
SopE	Yes	No	
SopE2	Yes	Yes	98.40%
SopF	Yes	Yes	92.70%
SptP	Yes	Yes	99.45%
SspH1	No	No	
StoD	No	No	
T3SS-1/2 effector proteins			
AvrA	Yes	No	
GogB	Yes	Yes	97.38%
GtgA	Yes	Yes	99.12%
GtgE	Yes	Yes	99.56%
PipB2	Yes	Yes	92.97%
SlrP	Yes	Yes	94.12%
SopD	Yes	Yes	99.17%
SseK1	Yes	Yes	98.83%
SpvC	Yes	Yes	85.16%
SpvD	Yes	Yes	99.07%
SteA	Yes	Yes	89.05%
SteB	Yes	Yes	53.56%
SteE	Yes	No	
T3SS-2 effector proteins			
PipB	Yes	Yes	97.59%
SifA	Yes	Yes	99.70%
SifB	Yes	Yes	98.44%
SopD2	Yes	Yes	98.75%
SsaB	Yes	Yes	99.25%
SpvB	Yes	Yes	99.66%
SrfJ	Yes	No	
SrgE	Yes	No	
SseF	Yes	Yes	95.77%
SseG	Yes	Yes	99.13%
SseI	Yes	Yes	89.75%
SseJ	Yes	Yes	100%
SseK2	Yes	No	
SseK3	Yes	No	
SseL	Yes	Yes	89.12%
SspH2	Yes	No	
SteC	Yes	Yes	96.38%

Western blotting data published in 2007 led to the hypothesis that whole genome sequencing would reveal significant variation between ST4/74 and SCSA50 (Paulin *et al.*, 2007). The sequence alignment of the predicted amino acid translations of T3SS effector proteins, however, demonstrated that many effector proteins are conserved between the strains (Table 3.1). SipC, the protein previously revealed to be differentially secreted by ST4/74 and SCSA50 (Paulin *et al.*, 2007), was surprisingly 100% conserved.

The T3SS proteins encoded by ST4/74 but not SCSA50 were AvrA, SopE, SteE, SrfJ, SrgE, SseK2, SseK3 and SspH2 (Table 3.1).

A recent computational approach probed 74 *S.* Choleraesuis genomes and found AvrA, SopE, SseK2 and SspH2 were absent in every genome (Rakov *et al.*, 2019). AvrA, SseK2, SseK3 and SteE function to dampen *Salmonella*-induced inflammation and cell death (Jones *et al.*, 2008; El qaidi *et al.*, 2017; Panagi *et al.*, 2020). SopE and SspH2, conversely, exert a pro-inflammatory response in eukaryotic cells (Mirold *et al.*, 1999; Bhavsar *et al.*, 2013). SrfJ and SrgE have had little study to define their function but are predicted to be involved in quorum sensing (Habyarimana *et al.*, 2014) and SCV stability (Kim *et al.*, 2009).

It is notable that half of the effectors missing from the genome of SCSA50 share a similar function to combat inflammation suggesting that *S.* Choleraesuis may have lost these effectors as it became host-adapted. In pigs, *S.* Choleraesuis causes less inflammation and damage to the intestinal epithelium than disease caused by *S.* Typhimurium (Paulin *et al.*, 2007), and therefore, the absence of a subset of anti-inflammatory effector proteins in SCSA50 may not be of critical importance.

Six effectors shared less than 90% sequence conservation including SopA, SpvC, SseI, SseL, SteA and SteB. Variation in effector identity was anticipated with SteA, SteB, SseL and SspH2 listed as three of the most

diverse between *S. enterica* serovars by multiple studies (Jennings *et al.*, 2017; Rakov *et al.*, 2019).

SopA is an important virulence factor secreted by T3SS-1 and contributes to bacterial invasion *in vitro* and enteritis *in vivo* (Wood *et al.*, 2000b; Raffatellu *et al.*, 2005) and is therefore, noteworthy that SCSA50 encodes a truncated SopA which should not be secreted by T3SS-1 (Higashide and Zhou, 2006). SCSA50 colonises the ileum of swine but infection does not typically result in intestinal inflammation or enteritis (Paulin *et al.*, 2007). Whether truncation of SopA influences the *in vivo* phenotype of SCSA50 must be further explored.

In contrast, the SCSA50 plasmid-encoded SpvC is 39 amino acids longer at the N-terminus than the protein encoded by ST4/74. SpvC promotes bloodstream dissemination of *S.* Typhimurium in a murine model (Gopinath *et al.*, 2019) and is therefore potentially a virulence factor relevant to the systemic disease caused by *S.* Choleraesuis. Functional biochemical characterisation of the SCSA50 protein would have to take place to ascertain any potential effect. Like SpvC, SseL is also longer in SCSA50, extended by 15 amino acids at the N-terminus.

The SteA N-terminal secretion signal (amino acids 1-10) (Cardenal-Muñoz and Ramos-Morales, 2011) is conserved between ST4/74 and SCSA50 but little is known about the functional domains of the protein itself. SteB is translocated by both T3SS-1 and -2 but its contribution to bacterial pathogenesis has not been fully defined.

SseI was previously mentioned as an important factor in systemic dissemination of *S.* Typhimurium ST313 by its pseudogene formation (Carden *et al.*, 2017). The SCSA50 SseI has a truncated N-terminus that is 30 amino acids shorter than ST4/74. The SseI secretion signal is predicted to be within the first 24 amino acids (Bhaskaran and Stebbins, 2012) and therefore, N-terminal truncation could indicate pseudogene formation in SCSA50.

This data emphasises that sequence data is important and has identified a series of differentially encoded effector proteins worthy of further research. The conservation of SipC, however, confirmed that sequence data alone cannot determine the *in vivo* pathogenesis of a strain.

It is important to consider that whilst genetic variation has undoubtedly been involved in serovar emergence and prediction (Lupolova *et al.*, 2017; Wheeler *et al.*, 2018; Rakov *et al.*, 2019), caution must be exerted in assigning a role to presence or absence of genes to serovar adaptation to a specific host since *in vivo* relevance must be determined. This huge complexity has impeded the understanding of the molecular mechanisms underlying host-adaptation of *Salmonella* serovars.

A multitude of factors may be involved including both bacterial virulence factors and the host itself (Smith, 1900).

3.3.2 Genomic prediction of type III secreted effector proteins

Genomic prediction of effector proteins using computational tools is a unique method of investigating host-pathogen interactions and numerous algorithms have been published to predict novel type III secreted virulence factors (Arnold *et al.*, 2009; Löwer and Schneider, 2009; Samudrala *et al.*, 2009; Yang *et al.*, 2010; Eichinger *et al.*, 2016). Several *S.* Typhi specific virulence factors have been identified from genome sequences including the Vi antigen, typhoid toxin and the recently discovered T3SS effector StoD (Raffatellu *et al.*, 2005; McDowell *et al.*, 2019). Thus, exploring the potential of serovar-specific virulence determinants in swine-adapted *S.* Choleraesuis is important.

Effector proteins often lack sequence similarity (McDermott *et al.*, 2011) which has confounded their identification by sequence alone. Recently published computational algorithms use homology to experimentally-confirmed effectors such as an N-terminal secretion signal or amino acid composition, chaperone binding domain, location in the genome or presence

of conserved motifs to identify putative type III secreted substrates (Karavolos *et al.*, 2005; Panina *et al.*, 2005; Arnold *et al.*, 2009; Löwer and Schneider, 2009; Samudrala *et al.*, 2009; Wang *et al.*, 2011; Büttner, 2012; Hui *et al.*, 2020).

To identify proteins predicted to be type III secreted in the genomes of ST4/74 and SCSA50, the EffectiveT3 computational tool was employed (Eichinger *et al.*, 2016). The online tool was one of the first resources to be developed for sequence-based prediction of effector proteins (Arnold *et al.*, 2009) and was found to be the more user-friendly compared to others (Yang *et al.*, 2010; Hobbs *et al.*, 2016; Hui *et al.*, 2020).

The output delivers a T3SS substrate score and identifies chaperone binding domains common to known effector proteins since most effectors are bound by a cytosolic chaperone which binds to a protein domain usually encoded in the first 150 amino acids of the protein to protect the effector from degradation prior to secretion via the T3SS needle (Stebbins and Galán, 2001).

The output data was first interrogated for experimentally-confirmed T3SS effectors to determine confidence in the software (Table 3.2).

Table 3.2 | Prediction of experimentally-confirmed T3SS effectors EffectiveDB

The genomes of ST4/74 and SCSA50 were loaded onto EffectiveDB and the programs EffectiveT3 (scored proteins more than 0.99 as predicted to be secreted) and EffectiveCCBD (identified chaperone binding domains) ran.

Effector	ST4/74			SCSA50	
	EffectiveCCBD	EffectiveT3	% Identity	EffectiveCCBD	EffectiveT3
T3SS-1 effector proteins					
SipA	-	1	99.60%	-	1
SipB	-	0.99	99.80%	-	1
SipC	-	1	100%	-	1
SipD	-	1	99.42%	-	1
SopA	LVGAINTIVN	1	59.90%	-	0.05
SopB	-	0.98	96.90%	-	1
SopE	LAKSILAVKN	0.46		Not encoded	
SopE2	FAKSITAVRN	1	98.40%	FAKSITAVRN	1
SopF	LSSDIQQVRN	0.078	92.70%	LSSDIQQVRN	0.5
SptP	-	1	99.45%	-	1
T3SS-1/2 effector proteins					
AvrA	-	0.92		Not encoded	
GogB	-	0	97.38%	-	0
GtgA	-	1	99.12%	-	1
GtgE	-	1	99.56%	-	1
PipB2	-	0	92.97%	-	0.063
SlrP	-	1	94.12%	-	1
SopD	-	0	99.17%	-	0
SseK1	-	0	98.83%	-	0
SpvC	-	1	85.16%	-	1
SpvD	-	1	99.07%	-	1
SteA	-	0.04	89.05%	-	0.04
SteB	-	0	53.56%	LSKSIRHISN	1
SteE	-	0		Not encoded	
T3SS-2 effector proteins					
PipB	-	1	97.59%	-	1
SifA	-	1	99.70%	-	1
SifB	-	0.002	98.44%	-	0.002
SopD2	-	0.9	98.75%	-	0.9
SsaB	-	0	99.25%	-	0
SpvB	-	0	99.66%	-	0
SrfJ	-	0.0058		Not encoded	
SrgE	-	1		Not encoded	
SseF	-	1	95.77%	-	1
SseG	IGNAVFYIAN	1	99.13%	IGNAVFYIAN	1
SseI	-	0.66	89.75%	-	0.96
SseJ	-	1	100%	-	1
SseK2	-	0		Not encoded	
SseK3	-	0.84		Not encoded	
SseL	-	1	89.12%	LALLIGEVEN	1
SspH2	-	0.92		Not encoded	
SteC	-	1	96.38%	-	0.99

A confident “type III secreted” EffectiveT3 score is higher or equal to 0.99, Such a score was assigned to only 50% of confirmed ST4/74 effectors and 59.4% of SCSA50 effectors (Table 3.2).

The data strengthened the theory that truncation of SopA in SCSA50 (Table 3.1) prevents its secretion, undoubtedly by loss of the chaperone binding domain that InvB binds (Table 3.2) (Higashide and Zhou, 2006). EffectiveT3 scored ST4/74 SopA as a confident 1 with chaperone binding domain LVGAINTIVN while SCSA50 SopA was scored 0.05 (Table 3.2).

As discussed previously in this chapter, variance in SseL sequence is prevalent across *S. enterica* serovars (Rakov *et al.*, 2019) and SCSA50 SseL is 15 amino acids longer than the protein encoded by ST4/74 (Table 3.1). This was, however, not the region where chaperone binding domain LALLIGEVEN was identified by EffectiveT3. The program could potentially have failed to identify a domain in ST4/74 SseL that chaperone SrcA binds (Cooper *et al.*, 2010), although recent data demonstrated that *S.* Typhimurium SseL is secreted without a functional SrcA under T3SS-2 inducing conditions (Godlee *et al.*, 2018).

Next, the predictive data generated from the genomes of ST4/74 and SCSA50 was used to identify potential hypothetical or putative proteins which could be secreted (Table 3.3).

Table 3.3 | Uncharacterised proteins annotated as confident T3SS effectors by EffectiveDB

These proteins were scored > 0.99 by EffectiveT3 and contained a chaperone binding domain (identified by EffectiveCCBD).

Annotations were accessed from the published genomes of ST4/74 and SCSA50 and BLASTP.

Uncharacterised protein	Annotation in ST4/74 or SCSA50	BLASTP identical protein groups
STM474_0307	Hypothetical protein	SymE family type I addiction module toxin
STM474_2285	Putative L-serine dehydratase	L-serine ammonia-lyase
STM474_2877	Putative cytoplasmic protein	SEC-C domain-containing protein
STM474_4513	Putative acetyltransferase	GNAT family N-acetyltransferase
STM474_4620	Putative DNA-binding protein	Transcriptional regulator ReiD
SCA50_0077	Hypothetical protein	Unnamed protein product
SCA50_2361	Putative D-serine dehydratase	L-serine ammonia-lyase
SCA50_2617	Putative inner membrane protein	Hypothetical protein
SCA50_3848	Putative transcriptional regulator	LacI family transcriptional regulator

Predictive profiling of the translated proteomes scored 606 ST4/74 and 584 SCSA50 proteins as potentially type III secreted (EffectiveT3 score 0.99 or above). Of those, five ST4/74 and four SCSA50 proteins that were annotated as hypothetical or putative also contained a chaperone binding domain (Table 3.3).

Several proteins, despite using the BLASTP resource on NCBI, remained hypothetical (STM474_2877, SCA50_0077, and SCA50_2617). Three proteins were potentially involved in metabolic pathways (STM474_2285, STM474_4513, and SCA50_2361) and two in transcriptional regulation (STM474_4620 and SCA50_3848). STM474_0307 shares homology with Syme-family toxins involved in the bacterial stress response (Kawano, Aravind and Storz, 2007).

Since *S.* Typhimurium has been subjected to extensive investigation, a variety of interrogatable datasets exist. Transposon directed insertion-site sequencing (TraDIS), for example, conducted after infection of cattle, pigs, and chickens with ST4/74 (Chaudhuri *et al.*, 2013) allowed hypothetical proteins to be tested *in vivo*. Insertions in STM474_2877 were found to be significantly attenuating in chickens, cattle and pigs while insertions in STM474_4620 was attenuating in pigs and cattle. Attenuation of STM474_4620 could be a result of mutation of a key metabolic pathway of myo-inositol utilisation that is required in pigs and cattle (Rothhardt *et al.*, 2014). Hypothetical STM474_2877, however, is an excellent uncharacterised candidate for future research.

This prediction strategy to identify novel effector proteins must be matched with experimental data. If these proteins were identified in the secretome, a lot more confidence could be given to these type III secreted candidates.

3.3.3 *In vitro* conditions to induce the *Salmonella* type III secretion systems

Host cell contact is critical for the secretion of *Yersinia* spp. and *Shigella flexneri* effectors (Darwin and Miller, 1999, 2001; Büttner, 2012) whilst the *S. enterica* T3SS-1 can readily be expressed *in vitro* (Shea *et al.*, 1996).

The conditions used to induce secretion associated with T3SS-1 were adapted from the previous literature (Davis and Mecsas, 2007; Hébrard *et al.*, 2011; Kröger *et al.*, 2013). Bacteria were cultured overnight with shaking at 30 ˚C in Luria-Bertani (LB) broth before subsequent subculture in fresh media at 37 ˚C for 4 hours to induce type III secretion of effector proteins into the culture supernatant. At 37 ˚C effectors associate with cognate chaperone proteins (Cossart and Sansonetti, 2004), ready for organised secretion by the T3SS apparatus.

Shifting the temperature of overnight cultures was first tested using stable single-copy green fluorescent protein (GFP) chromosomal fusion strains (Hautefort *et al.*, 2003) to measure the activity of the promoters of genes encoding the constitutively expressed housekeeping protein RpsM, T3SS-1 inner membrane protein PrgH, T3SS-2 needle SsaG and a promoterless negative control by using GFP production to infer gene expression.

The strains were cultured overnight at 30 ˚C, optical density (OD) normalised to 1.0 and diluted 1:20 to be cultured at either at 30 ˚C or 37 ˚C for four hours (Figure 3.2). During the experiment, 200 µl was removed to measure OD hourly (Figure 3.2B) and GFP production at the end of the culture (Figure 3.2A).

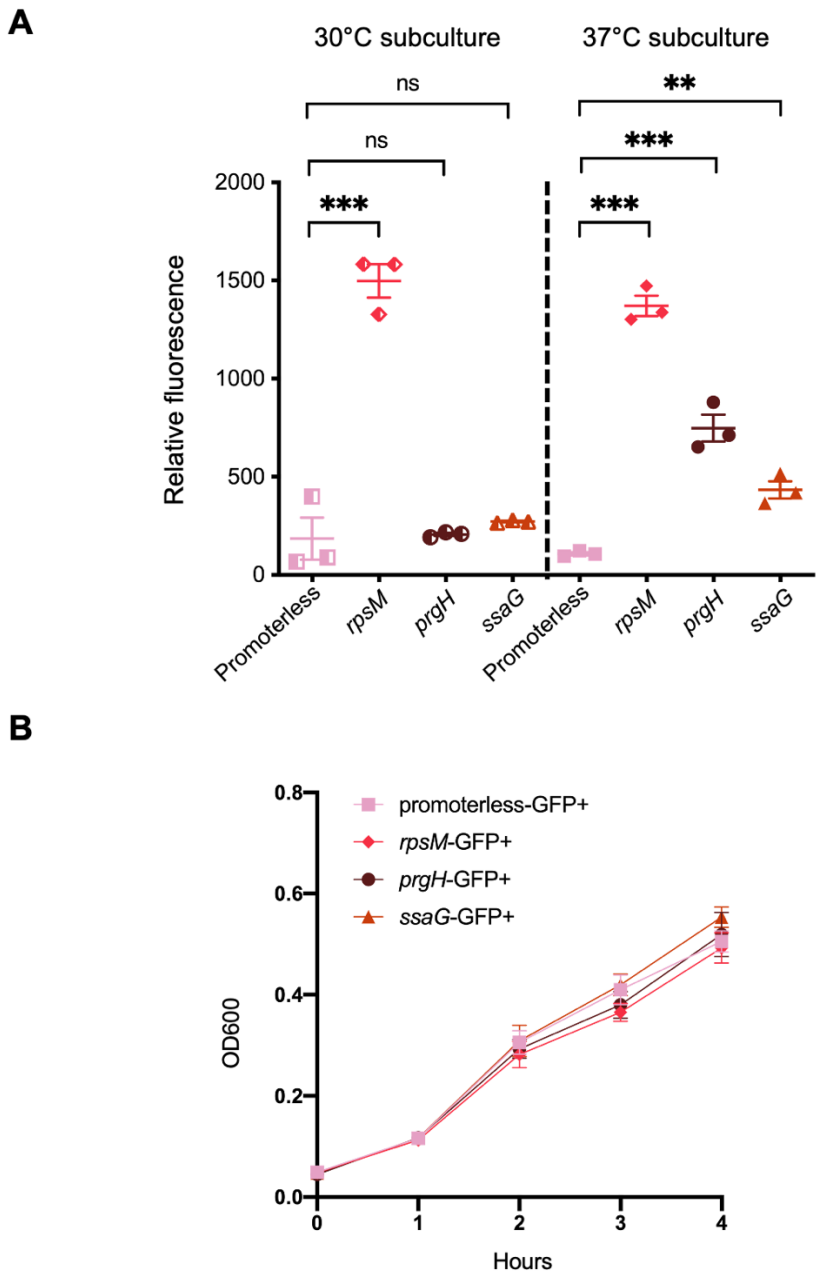


Figure 3.2 | Expression of single gene-GFP fusions under T3SS-1 inducing conditions.

(A) Expression of GFP was measured in *S. enterica* serovar Typhimurium strain SL1344 after 4 hours of subculture at 37℃ or 30℃. Experiments were performed with three biological replicates and the error bars are representative of the standard error of the mean. A student's t test was used to compare the values of genes of interest and the promoterless control.

(B) Three biological replicates of the SL1344 fusion strains were cultured at 37℃ in Luria-Bertani broth and optical density measured using a microplate reader every hour.

The conditions to induce T3SS-1 were confirmed using promoter-GFP chromosomal fusions which demonstrated that shifting the culture temperature to 37 °C induced the promoter activity of *prgH* and *ssaG* significantly, compared to the promoterless control (Figure 3.2A). Subculture at 30 °C did not result in significant promoter activity of either *prgH*-GFP or *ssaG*-GFP, suggesting that a temperature shift positively activated T3SS transcriptional expression.

Additionally, constitutively-expressed housekeeping gene *rpsM* did not significantly differ between conditions. Promoter activity of *ssaG* during culture in LB broth was an interesting discovery and suggested that T3SS-2 could also be induced by these conditions.

The expression of *ssaG in vitro* has previously been demonstrated to be stationary-phase specific in LB (Lim *et al.*, 2006) but overall, T3SS-2 associated genes are preferentially expressed under intracellular conditions (Valdivia and Falkow, 1997; Cirillo *et al.*, 1998; Eriksson *et al.*, 2003). The above data collected at 37 °C was not confounded by differential growth kinetics of the strains, as depicted in Figure 3.2B.

With the transcription of *prgH*-GFP demonstrated, it was expected that T3SS-1 inducing conditions induced the expression of other co-transcribed genes such as *prgHIJK* (Pegues *et al.*, 1995) and SPI-1 for *S.* Typhimurium since T3SS-1 central transcriptional activator HilA positively regulates *prgH* (Lostroh *et al.*, 2000; Lee, 2001).

Bacterial gene transcription is a tightly controlled process, especially within the complex environment of the host or even the nutrient-rich alkaline LB broth often used to mimic the host intestinal lumen where survival of the fittest is paramount (Kröger *et al.*, 2013). It is therefore curious that *ssaG*, a gene relevant within the acidic SCV and macrophage infection, would be

expressed under these conditions. Interestingly, *prgH* is repressed by the activity of two-component regulatory system PhoP/PhoQ (Pegues *et al.*, 1995) whilst the system activates *ssaG* transcription (Bijlsma and Groisman, 2005). It is important, however, to consider that transcription does not guarantee these genes are translated into proteins.

Changes in temperature has been well-studied with regards to the gene expression of mammalian and plant pathogens since 37 °C is associated with warm-blooded mammalian hosts (Steinmann and Dersch, 2013). Temperature change can alter transcription of genes within virulence networks by the introduction of conformational changes in DNA which alter the affinity of RNA polymerase binding (Nickerson and Achberger, 1995; Hurme and Rhen, 1998). Histone-like nucleoid-structuring protein (H-NS) is an important thermosensor which controls these conformational changes in *Salmonella* and represses gene expression when the temperature is below 37 °C (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). The data in figure 3.2A correspondingly demonstrates that temperature shift to 37 °C is more permissive to transcription of pathogenicity island genes *prgH* and *ssaG*.

To validate whether proteins were secreted into culture supernatants under these T3SS-1 inducing conditions, the supernatant was harvested and filter-sterilised, and proteins precipitated. Under T3SS-1 conditions both ST4/74 and SCSA50 secreted ~30 µg of protein which was precipitated using pyrogallol red-molybdate methanol (PRMM) (Caldwell and Lattemann, 2004).

1 µg of the total secreted proteins from each strain was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins strained with Coomassie Blue (Figure 3.3).

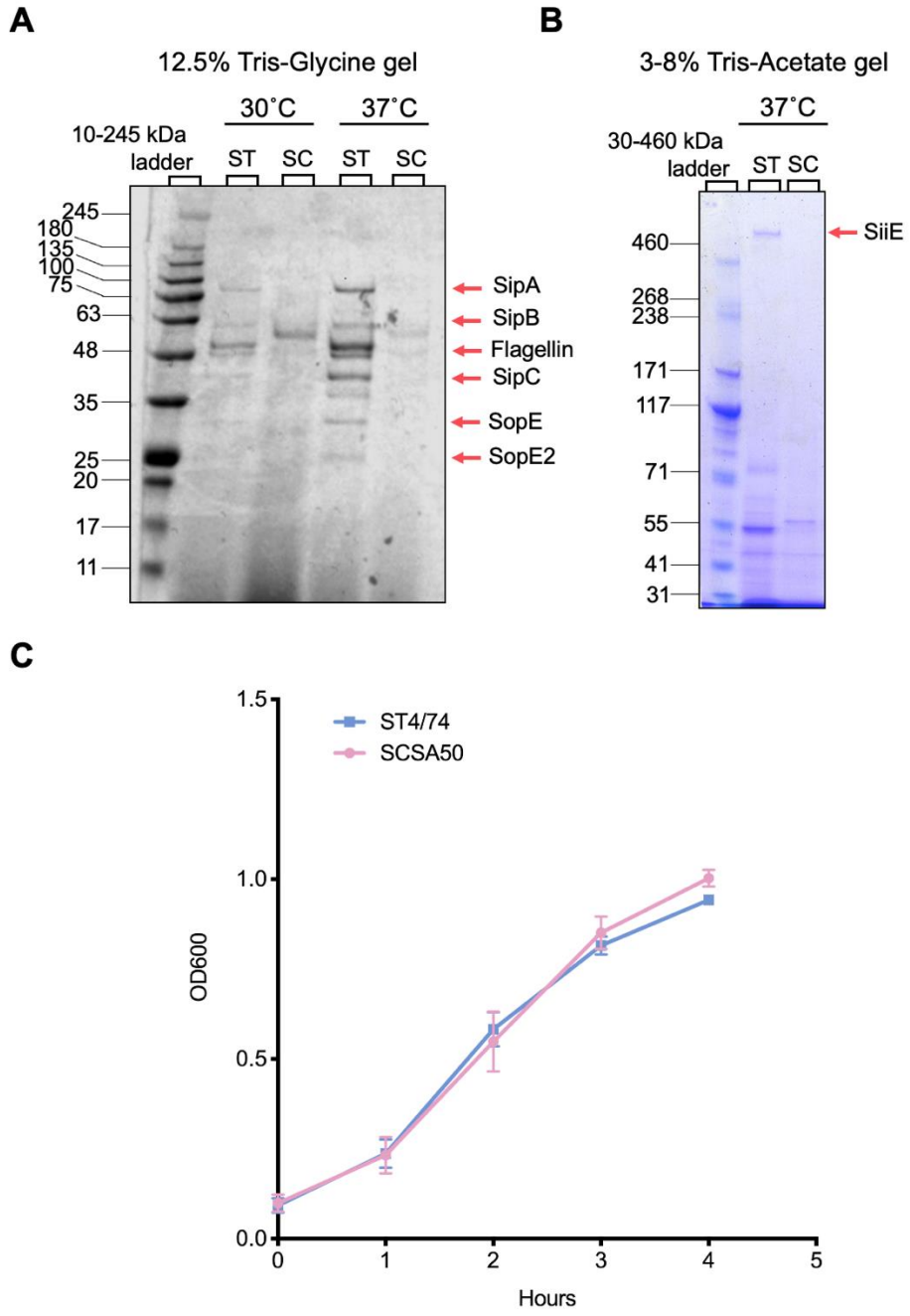


Figure 3.3 | Secretion profiles of ST4/74 and SCSA50

The secreted proteins by ST4/74 and SCSA50 were separated by SDS-PAGE and stained with Coomassie Blue.

(A) 12.5% Tris-Glycine gel to visualise proteins between 11 and 245 kDa.

(B) A 3-8% Tris-Acetate gel was used to visualise higher molecular weight proteins.

(C) Both strains had identical growth kinetics after subculture at 37°C (n=3) and therefore secretion was not affected by differential bacterial growth.

Separated by SDS-PAGE, the secretion profiles of both ST4/74 and SCSA50 under T3SS-1 inducing conditions was distinct, which supported the goal to deconvolute this comparative complexity using proteomics.

Several observations could be made from the strain-specific profiles. Despite loading an equal quantity of protein secreted by each strain under T3SS-1 conditions and a consistent pattern of growth (Figure 3.3C), SCSA50 had a less complex profile than ST4/74, which secreted more proteins upon 37 °C subculture than 30 °C (Figure 3.3A).

An easily identified protein was SiiE, the 595 kDa adhesin secreted by the type I secretion system encoded on SPI-4 (Morgan *et al.*, 2007) (Figure 3.3B). Despite both ST4/74 and SCSA50 encoding *siiE* and its cognate secretion system, the protein was at a visually detectable concentration in the ST4/74 secretome separated on a 3-8% Tris-acetate gel but not in the secretome of SCSA50 (Figure 3.3B). It is interesting to note that SPI-4 is co-ordinately regulated with SPI-1 by T3SS-1 transcriptional activator HilA (Main-Hester *et al.*, 2008) and that SPI-4 mutants were attenuating only in cattle but not pigs, suggesting that the adhesin could be lost during adaptation to a specific host (Morgan *et al.*, 2007).

This data supports the hypothesis that SCSA50 has a different secretion profile than ST4/74, despite equal growth kinetics and the quantity of protein secreted.

For greater understanding of pathogenesis, investigating the expression of virulence factors secreted by both T3SS-1 and -2 is important since both systems are important for infection and disease progression of salmonellosis *in vivo*, at least for *S.* Typhimurium (Carnell *et al.*, 2007; Chaudhuri *et al.*, 2013; Vohra *et al.*, 2019). Unlike the conditions that induce T3SS-1 secretion, triggering T3SS-2 secretion *in vitro* requires conditions akin to the SCV that is an acidic environment starved of phosphates and magnesium (Beuzón *et al.*, 1999; Deiwick *et al.*, 1999; Coombes *et al.*, 2004; Yu *et al.*, 2010).

Several strategies were employed to induce the secretion of T3SS-2 effectors into culture supernatants and the final protocol optimised from (Yu *et al.*, 2010).

The strains were cultured overnight at 37 °C in LB broth and washed three times in T3SS-2 minimal culture media (pH 5.8) before 1:20 culture for 6 hours at pH 5.8. The bacteria were then pelleted and resuspended in T3SS-2 minimal culture media at pH 7.2 overnight (Deiwick *et al.*, 1999; Yu *et al.*, 2004; Niemann *et al.*, 2011).

Secreted proteins in the bacterial supernatant were precipitated using StrataClean resin and prepared for SDS-PAGE separation and silver staining (Figure 3.4).

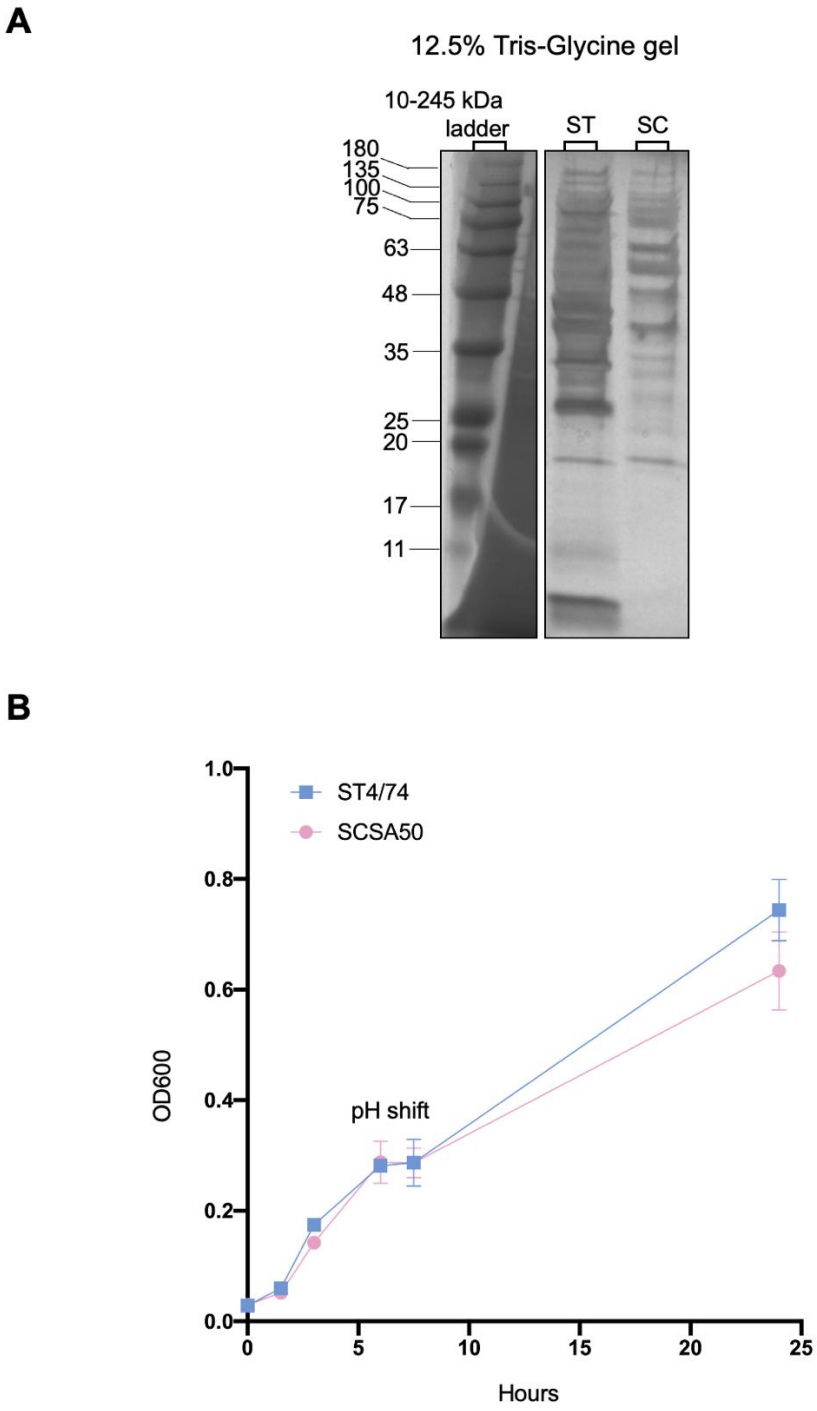


Figure 3.4 | The distinct T3SS-2 secretion profiles of ST4/74 and SCSA50

Strains were cultured under T3SS-2 minimal media acidic conditions for 6 hours before an overnight pH shift to 7.2 was performed.

(A) Proteins were precipitated using StrataClean resin and separated by SDS-PAGE before silver staining.

(B) Growth kinetics of bacteria grown under T3SS-2 conditions over 24 hours. The pH shift from pH 5.8 to pH 7.2 was performed at hour 6.

Using silver staining to detect as little as 1 nanogram of protein, the secretion profiles of ST4/74 and SCSA50 were distinct under T3SS-2 conditions (Figure 3.4A), similar to T3SS-1 conditions (Figure 3.3A). The profile of stained ST4/74 proteins is denser and more complex than SCSA50 and some bands of small proteins (< 20 kDa) differ in size (Figure 3.4A).

Since StrataClean resin was used to precipitate the secreted proteins, the total amount loaded could not be normalised. Loading was instead normalised by demonstrating equal growth kinetics under T3SS-2 inducing conditions (Figure 3.4B) and equivalent retrospective CFU counts.

Several studies have used *in vitro* T3SS-2 conditions to investigate the secretome, proteome and transcriptome of *S.* Typhimurium strains and lead to a greater understanding of the gene expression under simulated intracellular conditions (Shi *et al.*, 2009; Niemann *et al.*, 2011; Brown *et al.*, 2012; Kröger *et al.*, 2013). Data, however, has suggested that the intra-macrophage and T3SS-2 media transcriptome of *S.* Typhimurium are not identical, suggesting that host factors are an additional important pressure (Kröger *et al.*, 2013; Srikumar *et al.*, 2015).

The optimised culture condition to induce T3SS-2 secretion was then tested using single-copy *rpsM*, *prgH, ssaG* and promoterless GFP fusions (Hautefort *et al.*, 2003). As previously described, the fusion strains were cultured in T3SS-2 media at pH 5.8 for six hours and either shifted pH for 90 minutes or overnight (Figure 3.5).

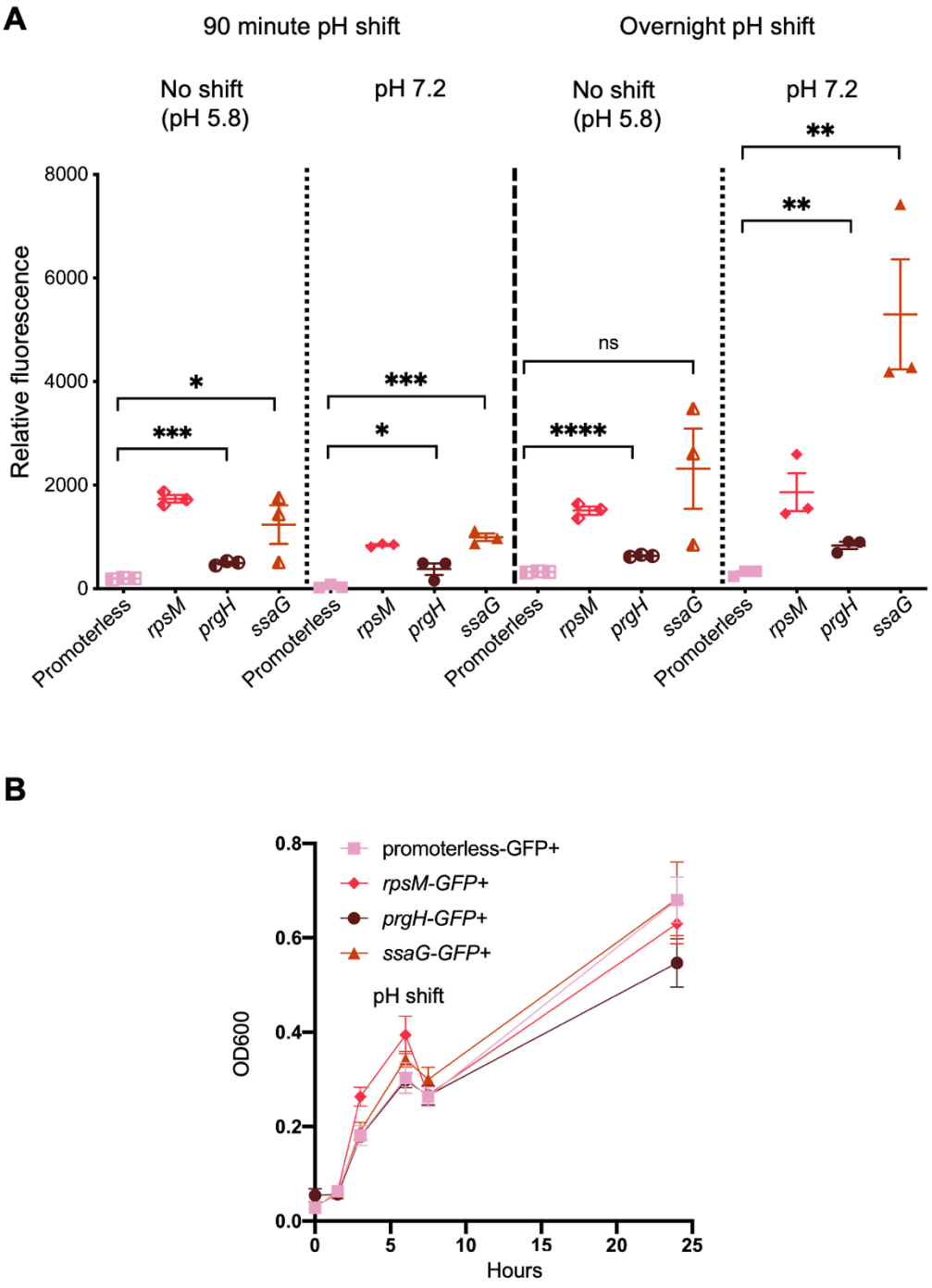


Figure 3.5 | Measurement of single gene-GFP fusions under T3SS-2 inducing conditions.

(A) Expression of GFP was measured after 7.5 hours (with or without 90 minute pH change from 5.8 to 7.2) and after 24 hours (with or without pH change). Experiments were performed with three biological replicates and the error bars are representative of the standard error of the mean. A student's t test was used to compare the values of genes of interest and the promoterless control.

(B) Three biological replicates of the SL1344 fusion strains were cultured under T3SS-2 conditions and optical density measured using a microplate reader.

With GFP production measured as a proxy for gene transcription, the expression of the T3SS apparatus components *prgH* (T3SS-1) and *ssaG* (T3SS-2) in T3SS-2 inducing conditions was evaluated (Figure 3.5A).

After 90 minutes shift to pH 7.2, the expression of both *prgH* and *ssaG* was significantly higher than the promoterless control despite lack of T3SS-2 secretion (data not shown). An overnight (~17 hours) shift to pH 7.2 also elicited a significant increase in *prgH* and *ssaG* expression.

The expression of T3SS-1 apparatus component was not surprising as late intracellular T3SS-1 activity has been well described in both macrophages and epithelial cells, and implicated in escape from the SCV into the host cytosol (Knodler *et al.*, 2014; Finn *et al.*, 2017). Without a shift to neutral pH the expression of *prgH* and *ssaG* was still significantly higher than a promoterless control. The constitutively expressed positive control housekeeping gene *rpsM* did not vary significantly between culture conditions.

It must be noted that environmental conditions have a substantial effect upon bacterial gene expression, which can change within minutes in response to environmental cues (Cho *et al.*, 2009; Mandlik *et al.*, 2011; Kröger *et al.*, 2012, 2013). The significant data at pH 5.8 is indicative of the power of the environmental conditions created by T3SS-2 media, mirroring several transcriptomic studies (Kröger *et al.*, 2013) but considering protein secretion was not abundant, it suggests that these studies could be missing key regulatory cascades and genes key to T3SS dependent intracellular survival.

Overall both T3SS-1 and T3SS-2 inducing conditions have been characterised and optimised and despite equivalent growth kinetics, ST4/74 and SCSA50 differed in their profiles of secreted proteins. This led to potential questions whether each strain responded differently to the same

environmental condition and whether profiling by proteomics would uncover major differences.

3.3.4 Shotgun proteomics identifies a repertoire of type III secreted proteins

Despite the importance of secreted effector proteins to *Salmonella* virulence, few studies have investigated the secreted proteins of serovars other than *S*. Typhimurium (Wood *et al.*, 1996, 2000a; Galyov *et al.*, 1997; Jones *et al.*, 1998; Paulin *et al.*, 2007; Pullinger *et al.*, 2007; Johnson *et al.*, 2018b), and none of these studies profiled the secretome.

Shotgun proteomics is a gel-free established tool to study bacterial virulence factors by protein digestion prior to analysis and identification by a high-resolution mass spectrometer (Zhu *et al.*, 2010).

The technique was used to deconvolute the secretion profiles of ST4/74 and SCSA50 under T3SS-1 inducing conditions. T3SS-1 conditions were preferentially chosen for a gel-free approach that could not be performed using T3SS-2 proteins bound to StrataClean resin. Additionally, T3SS-1 conditions stimulated secretion of approximately 3 µg/ml of culture.

The secreted proteins were trypsin-digested and prepared for electrospray ionisation quadropole time-of-flight mass spectrometry (ESI-QUAD-TOF-MS) under the supervision of Dr Dominic Kurian, manager of the Roslin Institute Proteomics and Metabolomics facility. The mass spectrometry was performed by Dr Kurian, who also performed the data analysis using the Matrix Science server.

The table below summarises the repertoire of proteins identified with confidence in the secretome of one biological replicate of each strain (Protein score > 13 and > 2 unique peptides) (Table 3.4).

Table 3.4 | Proteins secreted by ST4/74 and SCSA50 under T3SS-1 conditions detected by shotgun proteomics.

Protein	Description	ST4/74		SCSA50	
		Score	Peptide matches	Score	Peptide matches
Flagellar proteins					
FlgB	Flagellar basal-body protein	45	3		
FlgD	Flagellar hook assembly protein	825	15	1092	15
FlgE	Flagellar hook protein	135	4		
FlgF	Flagellar basal-body rod protein	122	3		
FlgG	Flagellar basal-body rod protein	371	8	123	2
FlgK	Flagellar hook-associated protein	5756	100	3014	56
FlgL	Flagellar hook-associated protein	8356	115	6528	73
FlgM	Negative regulator of flagellin synthesis	1873	29	2790	37
FliC	Phase-1 flagellin	11740	188	13904	164
FliD	Flagellar capping protein	11362	170	13690	151
FliE	Flagellar hook-basal-body complex protein	189	3		
FlijB	Flagellin	8739	147		
FliK	Flagellar hook-length control protein	1042	13	611	7
SPI-1 associated proteins					
OrgC	SPI-1 protein	1442	34	1936	27
InvJ	T3SS-1 needle regulator	2736	49	2705	34
PrgI	T3SS-1 apparatus protein	2418	29	1686	21
PrgJ	T3SS-1 apparatus protein	2459	36	2899	38
SipA	T3SS-1 effector	7506	129	4841	66
SipB	T3SS-1 effector	6310	88	3735	45
SipC	T3SS-1 effector	10915	179	7969	90
SipD	T3SS-1 effector	1274	44	393	10
SopA	T3SS-1 effector	200	5		
SopB	T3SS-1 effector	1142	34	1165	17
SopE	T3SS-1 effector	1126	24		
SopE2	T3SS-1 effector	113	6		
SptP	T3SS-1 effector	90	6		
SiiE	SPI-4 T1SS secreted adhesin	1720	45	65	4
SPI-1/2 associated proteins					
GogA	T3SS-2 effector	136	4		
GtgA	T3SS-1 and T3SS-2 effector			565	7
PipB2	T3SS-1 and T3SS-2 effector	51	2		
SlrP	T3SS-1 and T3SS-2 effector	57	4	331	6
SteA	T3SS-1 and T3SS-2 effector			1453	21
SPI-2 associated proteins					
SseL	T3SS-2 effector			158	2
Uncharacterised proteins					
SCA50_0733	Sel1 repeat family protein YbeQ			24	2

Table 3.4 cont.

Protein	Description	ST4/74		SCSA50	
		Score	Peptide matches	Score	Peptide matches
Bacteriophage proteins					
HdpD	Bacteriophage head decoration protein			141	6
SCA50_0382	Bacteriophage coat protein			308	6
Possible products of cell lysis					
GroEL	Protein folding chaperonin			697	13
YdhC	Multidrug efflux transporter	29	2		
TufA	Elongation factor			192	3
GapA	Glyceraldehyde-2-phosphate dehydrogenase A	50	3		
MalE	Maltose-binding protein			131	2
Mdh	Maltate dehydrogenase	137	2	222	2
PgK	Phosphoglycerate kinase			107	2
YjgF	2-iminobutanoate/2-iminopropanoate deminase	50	3	156	5

Importantly, this data confirmed that T3SS-1 conditions induced expression of T3SS-1 in both ST4/74 and SCSA50, with both needle complex components and substrate effector proteins identified (Table 3.4). The score highlighted in Table 3.4 is the protein score which is calculated from matching spectral data to a protein sequence. A higher protein score is a more confident match.

The unique peptide matches, which belong to only one protein in the respective database searched, also indicates protein identification confidence as more unique peptides confers a more confident protein match. Since quantitative data could not be extracted from this experiment, the repertoire of secreted proteins were compared between the two strains.

Mass spectrometry identified 53 and 54 proteins in the secretome of ST4/74 and SCSA50, respectively. Of those, only 34 and 31 had two or more unique peptide matches and are listed in Table 3.4.

For both strains the proteins scored with the most confidence were flagellar components, which aligns with previous proteomic studies which demonstrated that flagellar proteins are the most abundant secreted proteins (Niemann *et al.*, 2011). The bacterial flagellum is itself a T3SS that spans the inner and outer cell membrane, secreting the extracellular flagellar proteins that form the flagellum hook (FlgL, FlgK, FlgD, and FlgE) and filament (FliD and FliC) (Diepold and Armitage, 2015). The abundance of flagellar proteins has been noted as a contaminant in secretome studies, and flagellin mutants have been previously employed in order to detect low-abundant proteins (Brown *et al.*, 2012).

As expected, all six secreted flagellar proteins (FliD, flagellin (FliC or FlijB), FlgL, FlgK, FlgD, and FlgE) were identified in the secretomes of ST4/74 while five were identified in the SCSA50 secretome except FlgE, the hook subunit, which was not identified. No outer membrane, inner membrane or cytoplasmic proteins of the flagellum were identified but four periplasmic

components (FlgB, FlgD, FlgF, and FliE) were present in the ST4/74 secretome. FlgD was also found in the SCSA50 data.

Extracellular components of the T3SS-1 apparatus including PrgI, PrgJ, and InvJ were also detected in both strains. Nine T3SS-1 preferential effector proteins were detected in the ST4/74 dataset and four were missing from the secretome of SCSA50 including SopA, SopE, SopE2, and SptP. As presented previously in this chapter, SCSA50 encodes a truncated SopA which lacks the N-terminal secretion signal and does not encode SopE.

Another meaningful T3SS-1 associated protein is SiiE, which was not detected by Coomassie Blue staining in the secretion profile of SCSA50 under T3SS-1 conditions (Figure 3.3) but was identified by mass spectrometry in datasets of both strains. Of note, however, is that the protein score for ST4/74 SiiE was 1720 while that of SCSA50 SiiE was 65, suggesting that it was identified with much less confidence. Both strains secreted three effectors which are either T3SS-1 or -2 substrates and one T3SS-2 preferential effector was detected only in the SCSA50 sample.

In summary, this experiment identified fourteen T3SS effector proteins which is a repertoire similar to other studies, 12 of which were identified by T3SS-1 induction and quantitative proteomics (Cheng *et al.*, 2017) and 21 detected under T3SS-2 profiling (Niemann *et al.*, 2011).

One putative protein, YbeQ that is a Sel1 repeat family protein with no known function, was also identified in the SCSA50 secretome. Two bacteriophage proteins were identified in the SCSA50 secretome related to head decoration and the phage coat. It is not unusual for phage proteins to be detected in the secretion profile of bacteria since filamentous phages can hijack bacterial secretion pathways to assembly and exit their host (Gagic *et al.*, 2016).

Other proteins identified were non-classical secreted proteins related to protein stability, metabolism and the outer membrane such as GroEL, YdhC, TufA, GapA, MalE, Mdh, Pgk, and YjgF. Apart from YdhC, these proteins

have all previously been identified to confirm unavoidable minimal cell lysis in *S. enterica* proteomics experiments (Niemann *et al.*, 2011; Cheng *et al.*, 2017).

Whilst quantitative data could not be extracted from this experiment, the differential protein scores for several type III secreted effectors are of great interest for future work. The SCSA50 T3SS-1 effector SipD score, for example, was 70% less than the ST4/74 SipD score whilst the ST4/74 T3SS-1 or -2 effector SlrP protein score was 83% less than SCSA50. These results, together with the distinct SDS-PAGE secretion profiles (Figure 3.3A), re-iterated that a quantitative proteomic approach would be essential to deconvolute the secretomes of ST4/74 and SCSA50.

3.3.5 FlgK as a normalisation protein

The shotgun proteomic data was then used to inform on a quantitative label-free approach to quantify differences in effector secretion between ST4/74 and SCSA50 since the T3SS-1 conditions were considered robust (Table 3.4).

It was decided that a protein would be used to retrospectively normalise the quantitative proteomic experiment. FlgK is a secreted component of the bacterial flagellum, the hook-associated protein (Homma *et al.*, 1985), and was chosen as a normalisation protein. The protein was detected in the secretome of both strains by shotgun mass spectrometry (Table 3.4) and is considered highly conserved across *S. enterica* serovars (Yeh *et al.*, 2018). Between ST4/74 and SCSA50, the predicted translated sequence was 100% conserved.

Motility is inherently a *S. enterica* virulence strategy which contributes to epithelium invasion (Winter *et al.*, 2009; Wolfson *et al.*, 2020) and intestinal inflammation (Schmitt *et al.*, 2001) by agonising the innate immune receptor Toll-like receptor 5 (TLR5) (Hayashi *et al.*, 2001). FlgK, and the bacterial motility it contributes to, must be investigated between the strains.

To assess the use of FlgK as a normalisation protein, the transcription of *flgK* under T3SS-1 inducing conditions and the motility of the strains were measured (Figure 3.6).

In the absence of an antibody reactive to both ST4/74 and SCSA50 FlgK proteins, the transcription of the *flgK* gene was assessed by RT-qPCR (Figure 3.6A). qPCR was performed on cDNA synthesised from RNA extracted from bacteria under T3SS-1 inducing conditions. The data was normalised to the expression levels of housekeeping gene *rpoD* and analysed using the Pfaffl method, as detailed in the relevant Materials and Methods section (Pfaffl, 2001).

Motility assays were then performed after T3SS-1 induction using 0.3% LB agar. The swimming diameter was measured after overnight static incubation, using *S.* Gallinarum strain SG9 as a non-motile negative control (Figure 3.6).

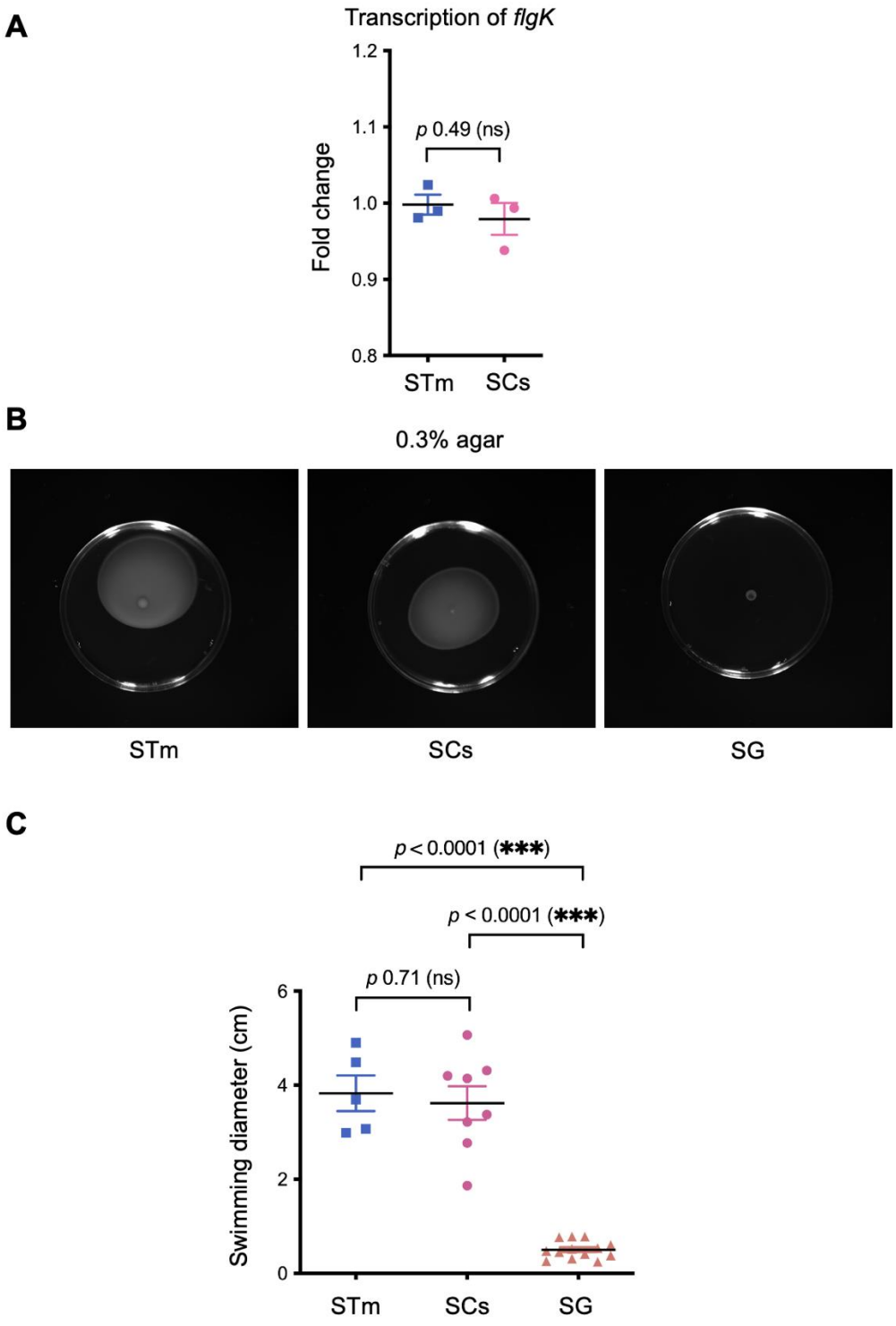


Figure 3.6 | Transcription of *flgK* and swimming motility of ST4/74 and SCSA50

(A) Transcription of *flgK* by ST4/74 and SCSA50 was assessed by RT-qPCR. During T3SS-1 inducing conditions, RNA was extracted after overnight culture and after 4 hour subculture. After DNase treatment and cDNA synthesis, transcript abundance was probed using quantitative PCR and the data analysed using the Pfaffl method (Pfaffl, 2001).

(B/C) Bacterial swimming motility was tested under T3SS-1 conditions using 0.3% soft agar. *S.* Gallinarum strain SG9 was used as a non-motile negative control.

Transcription of *flgK* was not significantly different between ST4/74 and SCSA50 (Figure 3.6A). In this experiment, mRNA transcripts were used to infer eventual protein concentration. There was also no significant difference in swimming motility between ST4/74 and SCSA50, and both strains were significantly more motile than aflagellate *S.* Gallinarum strain SG9 (Figure 3.6B/C).

Inter-serovar motility studies have previously demonstrated differences in motility between typhoidal serovars *S.* Typhi *S.* Paratyphi A and *S.* Sendai and *S.* Typhimurium (Elhadad *et al.*, 2015), but this was not observed between SCSA50 and ST4/74 (Figure 3.6B/C).

Since FlgK is critical for the establishment of the flagellum it is therefore critical for motility (Kutsukake and Ide, 1995). FlgK, and the bacterial motility it contributes to, was determined to be statistically insignificant between the strains and therefore considered appropriate for normalisation.

3.3.6 Label free quantitative proteomics reveals major differences in effector protein secretion between ST4/74 and SCSA50

As the conditions for T3SS-1 gene expression and protein secretion had been established, a quantitative proteomic approach was applied to characterise the secretomes of ST4/74 and SCSA50 from three independent experiments.

The samples were filter sterilised, quantified with the protein content estimated at approximately 100 µg/sample before sending to life sciences company Gemini Biosciences Ltd for label free quantitative proteomics and data analysis (https://www.geminibioscience.com/). Samples were subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Preliminary analysis determined that all three biological replicates of ST4/74 were of high quality but only one replicate of SCSA50 met quality control determinants. The three ST4/74 datasets was combined and downstream statistical analysis performed between the average ST4/74 and single SCSA50 data.

The analysis identified 367 proteins in total and the calculated fold changes of 272 were considered statistically significant ($p < 0.05$). Each protein was manually identified using tBLASTn since several genes in the genomes of ST4/74 and SCSA50 were misannotated. T3SS effector SteB, for example, was annotated as Dpr, a putative dipicolinate reductase, in SCSA50.

The mis-annotation additionally resulted in several proteins being duplicated during the downstream data analysis. In these instances, the fold change with the lowest *p* value was chosen to be included. The distribution of identified proteins was visualised by volcano plot with intercept lines dividing significant (Log_{10} p <0.05) fold changes (Log_2 fold change 2 or -2) and T3SS-associated proteins were highlighted (Figure 3.7).

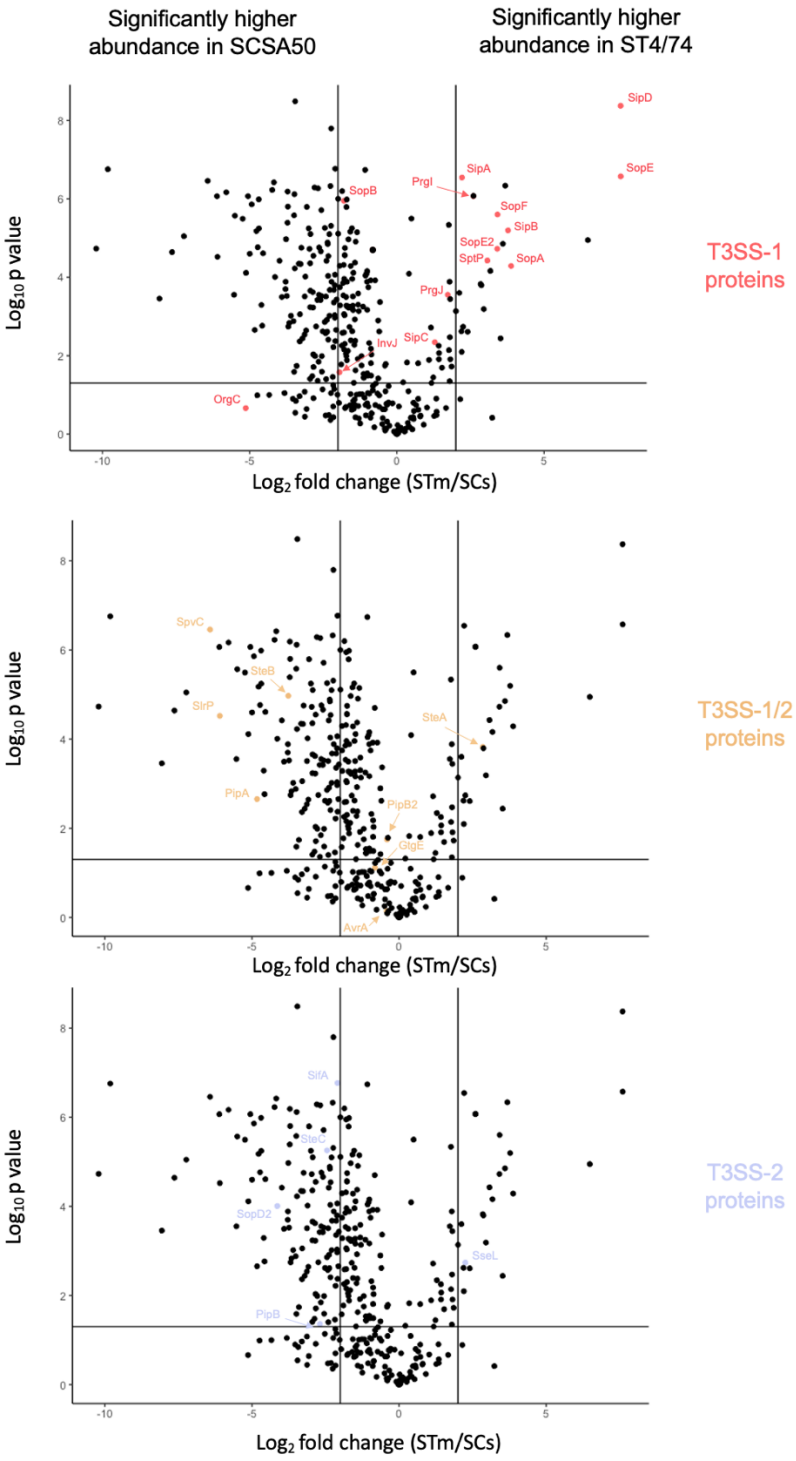


Figure 3.7 | The distrubtion of proteins identified by quantitative proteomics under T3SS-1 inducing conditions.

The distribution of fold changes between ST4/74 (STm) and SCSA50 (SCs) proteins identified by quantitative label-free proteomics. Proteins were transformed and plotted to

visualise the distribution of the dataset using ggplot2. X-axis vertical lines at -2 and 2 represent the Log2 fold changes within which proteins were considered signifcantly different between ST4/74 and SCSA50. The horizontal Y-axis line signfies the statistical signficance of the fold changes with proteins. T3SS effector proteins are highlighted.

25 proteins were higher in abundance in the secretome of ST4/74 whilst 119 proteins were secreted at higher levels by SCSA50 (Figure 3.7). There were 113 non-differential proteins (Figure 3.7). The plotted data clearly illustrated an uneven distribution of proteins, demonstrating that secretion of proteins is different between the strains and confirming previous observations (Figure 3.3).

A large number of differentially expressed proteins is not unique to ST4/74 and SCSA50 with an inter-serovar whole-cell proteome study reporting similarly larger numbers of differentially expressed proteins between *S.* Typhimurium, *S.* Enteritidis, *S.* Typhi and *S.* Paratyphi A (Saleh *et al.*, 2019).

In addition to the number of differential proteins between the strains, an important observation was the differential distribution of T3SS proteins (Figure 3.7). Thirteen T3SS-1 associated proteins had fold changes with significant confidence and nine were higher in abundance in the secretome of ST4/74. The remaining four proteins that scored between -2 and 2 were not considered differentially secreted. An additional secreted protein, OrgC, which is involved in assembly of the T3SS-1 apparatus (Kato *et al.*, 2018), was detected but without significant enrichment in either ST4/74 or SCSA50. Enrichment of T3SS-1 proteins in the secretome of ST4/74 was anticipated after western blot evidence of the differential secretion of T3SS-1 translocon SipC (Paulin *et al.*, 2007).

A surprising result, however, was that the pattern of effectors secreted by either T3SS-1 or T3SS-2 and those specific to T3SS-2 was the opposite – more of these proteins were in higher abundance in the secretome of SCSA50. Of the eight detected effectors secreted by either system, SteA was

the only protein enriched in the ST4/74 dataset whilst the abundance of four effectors (PipA, SlrP, SteB, and SpvC) was biased toward the SCSA50 secretome. Proteins GtgE and AvrA were detected, but their fold change was not significant. Of the five T3SS-2 proteins identified, one protein was biased toward ST4/74 and the remaining four were biased toward SCSA50.

The proteins which had Log_2 fold changes scored between -2 and 2 were apparatus proteins InvJ and PrgJ, and effectors SipC and SopB. The secreted needle component of the T3SS-1 apparatus, PrgI, was, however, enriched in the secretome of ST4/74. Since PrgJ is also a component of the injectisome as the inner rod which connects the needle PrgI to the apparatus membrane-spanning structure (Park *et al.*, 2018), this suggests either that the fold change limits were too strict to identify all enriched proteins or that PrgJ is not traditionally secreted and was present in both secretomes as membranous contamination.

Electron microscopy imaging has illustrated that *S.* Typhimurium expresses between 10 and 100 T3SS-1 needle complexes per cell (Kubori *et al.*, 1998) but there is a scarcity of data regarding T3SS needle complex formation and translocation dynamics information from non-typhoidal *Salmonella* serovars besides *S.* Typhimurium. This suggests that SCSA50, a host-adapted serovar, could express less T3SS-1 needle complexes than ST4/74 despite equal growth kinetics.

This differential bias between T3SS-1 and T3SS-2 secreted proteins has not been reported before, which adds novelty to this comparative study. A summary of the significant virulence factors as well as the differentially secreted flagellar, membrane, phage, metabolic and potential lysis products were compiled (Table 3.5) while the raw data is presented in the Appendix.

Table 3.5 | Proteins confidently identified by mass spectrometry in the secretome of ST4/74 and SCSA50.

T3SS-associated proteins, uncharacterised proteins and virulence factors are listed.

All other proteins are listed if their Log_2 fold change was below -2 or above 2.

Protein	Description	Log_2 fold change (STm/SCs)	Serovar bias	P value
T3SS-1 associated proteins				
PrgI	T3SS-1 apparatus	2.60	More in STm	8.54E-07
PrgJ	T3SS-1 apparatus	1.73		2.80E-04
InvJ	T3SS-1 apparatus	-1.95		2.65E-02
SipA	T3SS-1 translocon/effector	2.21	More in STm	2.86E-07
SipB	T3SS-1 translocon/effector	3.78	More in STm	6.37E-06
SipC	T3SS-1 translocon/effector	1.29		4.55E-03
SipD	T3SS-1 translocon/effector	7.60	More in STm	4.24E-09
SopA	T3SS-1 effector	3.88	More in STm	5.16E-05
SopB	T3SS-1 effector	-1.80		1.12E-06
SopE	T3SS-1 effector	7.60	More in STm	2.67E-07
SopE2	T3SS-1 effector	3.41	More in STm	1.88E-05
SopF	T3SS-1 effector	3.42	More in STm	2.51E-06
SptP	T3SS-1 effector	3.07	More in STm	3.74E-05
T3SS-1 or T3SS-2 associated proteins				
PipA	T3SS-1/2 effector	-4.83	More in SCs	2.21E-03
PipB2	T3SS-1/2 effector	-0.41		1.79E-02
SlrP	T3SS-1/2 effector	-6.09	More in SCs	3.01E-05
SpvC	T3SS-1/2 effector	-6.43	More in SCs	3.48E-07
SteA	T3SS-1/2 effector	2.85	More in STm	1.50E-04
SteB	T3SS-1/2 effector	-3.77	More in SCs	1.07E-05
T3SS-2 associated proteins				
PipB	T3SS-2 effector	-2.69	More in SCs	4.37E-02
SifA	T3SS-2 effector	-2.09	More in SCs	1.71E-07
SopD2	T3SS-2 effector	-4.14	More in SCs	9.84E-05
SseL	T3SS-2 effector	2.25	More in STm	1.83E-03
SteC	T3SS-2 effector	-2.45	More in SCs	5.54E-06
Uncharacterised proteins				
SCA50_0346	Hypothetical protein	-7.64	More in SCs	2.28E-05
SCA50_0392	Hypothetical protein	-4.93	More in SCs	1.38E-06
SCA50_2891	Hypothetical protein	-0.58		4.30E-04
STM474_2071	Hypothetical protein	-2.22	More in SCs	1.30E-04
STM474_2074	DUF2303 family protein	-5.06	More in SCs	8.56E-07
STM474_3876	Rhodanese-like domain-containing protein	-3.40	More in SCs	1.82E-02
YdgH	DUF1471 family protein	-1.41		8.56E-07
YgaM	Conserved hypothetical protein	-3.47	More in SCs	5.95E-05
Other secreted virulence factors				
AcnB	Peroxidase	-1.79		2.56E-03
BtuE	Peroxidase	-2.55	More in SCs	2.57E-02
Eco	Neutrophil elastase inhibitor	-2.78	More in SCs	1.03E-02
FimA	Type I fimbriae component	0.41		8.13E-05
KatE	Peroxidase	-5.50	More in SCs	2.7E-06
KatG	Peroxidase	-3.78	More in SCs	1.30E-04
PagC	Virulence membrane protein	-1.70		1.04E-06
SiiE	Giant adhesin	-0.82		2E-05
SodB	Superoxidase dismutase	-1.70		1.30E-04
SodC1	Superoxidase dismutase	-1.85		5.51E-03

Table 3.5 | cont.

Protein	Description	Log_2 fold change (STm/SCs)	Serovar bias	P value
Differential flagellar or chemotaxis proteins				
CheW	Chemotaxis protein	2.19	More in STm	2.40E-03
FlgB	Flagellar basal-body protein	2.12	More in STm	2.50E-04
FlgG	Flagellar basal-body rod protein	3.16	More in STm	6.91E-05
FlgL	Flagellar hook-associated protein	6.49	More in STm	1.13E-05
FliD	Flagellar capping protein	-3.46	More in SCs	3.26E-09
Differential membrane proteins				
EcnB	Membrane lipoprotein	-3.11	More in SCs	9.62E-05
LppA	Outer membrane lipoprotein	-3.78	More in SCs	2.00E-04
MetQ	Methionine-binding lipoprotein	-2.06	More in SCs	2.21E-03
MglB	Galactose-binding protein	2.01	More in STm	7.30E-04
MppA	Murein tripeptide transporter subunit	-6.11	More in SCs	8.57E-07
OmpA	Outer membrane protein A	-3.06	More in SCs	1.61E-06
OmpF	Outer membrane protein F	-2.41	More in SCs	3.10E-04
OmpX	Outer membrane protein X	-2.80	More in SCs	5.14E-07
PhoN	Membrane acid phosphatase	-2.34	More in SCs	9.25E-05
Pal	Peptidoglycan-associated protein	-2.96	More in SCs	4.40E-05
PstS	Phosphate-binding protein	-5.80	More in SCs	6.81E-07
YbaY	YbaY family lipoprotein	-4.72	More in SCs	1.72E-05
YbjP	Lipoprotein	-3.71	More in SCs	4.07E-06
YfiO	BAM complex protein (BamD)	-3.66	More in SCs	1.47E-03
YqjD	Inner membrane protein	-3.49	More in SCs	2.64E-06
Differential phage proteins				
HdpD	Head decoration protein	-7.23	More in SCs	8.98E-06
Stf	Tail fiber protein	-4.77	More in SCs	6.65E-06
SCA50_0381	Scaffold protein	-3.60	More in SCs	9.60E-04
SCA50_0382	Coat protein	-9.82	More in SCs	1.76E-07
SCA50_0390	Injection protein	-3.91	More in SCs	3.20E-04
STM474_2045	Tail sheath protein	2.20	More in STm	8.03E-03
STM474_2705	Tail-like protein	-3.68	More in SCs	1.81E-03
STM474_2706	Prophage VmtV	-5.00	More in SCs	2.52E-05
STM474_2712	Head protein	-5.24	More in SCs	3.21E-06
STM474_2714	Head-tail connector protein	-3.71	More in SCs	6.51E-07
Differential metabolic enzymes				
AceE	Pyruvate dehydrogenase complex protein	-2.23	More in SCs	1.6E-08
AceF	Pyruvate dehydrogenase complex protein	-2.93	More in SCs	7.92E-06
AdhE	Aldehyde-alcohol dehydrogenase	-2.84	More in SCs	2.83E-05
AdhP	Alcohol dehydrogenase	-3.13	More in SCs	3.10E-04
AtpF	ATPase synthase subunit	-2.83	More in SCs	1.95E-02
CadA	Lysine decarboxylase	-4.68	More in SCs	5.70E-06
CysK	Cysteine synthase	-3.00	More in SCs	5.67E-06
DapD	Lysine biosynthesis pathway protein	2.95	More in STm	6.50E-04
DeoD	Purine phosphorylation protein	-5.52	More in SCs	2.80E-04
FabA	Fatty acid biosynthesis pathway protein	-2.24	More in SCs	4.88E-06
FabB	Fatty acid biosynthesis pathway protein	-2.40	More in SCs	3.97E-02
FabZ	Fatty acid biosynthesis pathway protein	2.60	More in STm	8.40E-07

Table 3.5 | cont.

Protein	Description	Log_2 fold change (STm/SCs)	Serovar bias	P value
Differential metabolic enzymes (cont.)				
FbaB	Fructose aldolase	-2.14	More in SCs	4.40E-04
GapA	G3P dehydrogenase	-2.68	More in SCs	5.40E-07
GcvP	Glycine dehydrogenase	-2.16	More in SCs	6.07E-03
GlmM	Phosphoglucosamine mutase	-4.23	More in SCs	5.93E-07
GrxB	Glutaredoxin	-3.27	More in SCs	4.58E-05
GstA	Glutathione S-transferase	2.40	More in STm	2.46E-03
IcdA	Isocitrate dehydrogenase	3.60	More in STm	1.40E-05
MalE	Maltose binding protein	-4.54	More in SCs	2.45E-05
MalM	Maltose uptake protein	-2.57	More in SCs	1.94E-06
MlaC	Phospholipid-binding protein	-2.64	More in SCs	1.75E-05
PepT	Peptide catabolism protein	-2.42	More in SCs	2.69E-03
Pgm	Phosphoglucomutase	-2.33	More in SCs	3.90E-03
Pta	Phosphate acetyltransferase	-3.08	More in SCs	2.27E-03
PykA	Pyruvate kinase	-2.94	More in SCs	2.20E-04
SucB	L-lysine degradation pathway protein	-2.26	More in SCs	4.72E-07
YadF	Carbonate dehydratase	-2.35	More in SCs	1.43E-05
YghA	Oxidoreductase	-2.75	More in SCs	1.80E-03
YgjR	Oxidoreductase	-4.18	More in SCs	3.80E-07
YqhD	Alcohol dehydrogenase	-3.70	More in SCs	1.58E-06
STM474_4723	Aldehyde dehydrogenase	2.87	More in STm	1.60E-04
Differential possible products of cell lysis				
ClpB	Protein chaperone	-2.14	More in SCs	2.10E-04
ClpP	Protease subunit	-2.04	More in SCs	3.70E-04
CsrA	Translational regulator	-2.87	More in SCs	3.33E-02
DnaK	DNA chaperone	-2.38	More in SCs	1.22E-03
EttA	Translational throttle protein	-3.74	More in SCs	3.00E-04
Frr	Ribosome recycling factor	-2.29	More in SCs	9.60E-04
FusA	Elongation factor G	-2.28	More in SCs	2.10E-04
FkpA	Protein folding	-8.07	More in SCs	3.50E-04
HtpG	Protein chaperone	-3.29	More in SCs	8.80E-04
IhfA	Integration host factor	-2.11	More in SCs	1.60E-04
Lrp	Transcriptional regulator	3.68	More in SCs	4.62E-07
LysR	Transcriptional regulator	-2.94	More in SCs	5.80E-04
MsrA	Protein repair enzyme	-3.30	More in SCs	4.32E-03
NusB	Transcription antitermination protein	-2.11	More in SCs	9.30E-04
OsmE	Transcriptional activator	-3.51	More in SCs	1.32E-03
PhoP	Transcriptional regulator	-10.21	More in SCs	1.86E-05
Pnp	Polyribonucleotide nucleotidyltransferase	-2.59	More in SCs	3.80E-04
RaiA	Translation inhibitor	-2.29	More in SCs	7.98E-06
RecA	DNA repair	-4.60	More in SCs	5.10E-04
RihA	Ribonucleoside hydrolase	-3.49	More in SCs	2.59E-02
RmuC	DNA stabilisation protein	-2.86	More in SCs	2.40E-04
RplB	50S ribosomal protein L2	-3.21	More in SCs	3.63E-03
RplC	50S ribosomal protein L3	-2.97	More in SCs	1.89E-05

Table 3.5 | cont.

Protein	Description	Log_2 fold change (STm/SCs)	Serovar bias	P value
Differential possible products of cell lysis (cont.)				
RplE	50S ribosomal protein L5	-2.10	More in SCs	1.08E-03
RplF	50S ribosomal protein L6	-2.59	More in SCs	2.18E-05
RplN	50S ribosomal protein L14	-3.25	More in SCs	1.77E-05
RplR	50S ribosomal protein L18	-2.36	More in SCs	1.30E-05
RplT	50S ribosomal protein L20	-2.56	More in SCs	1.03E-02
RplU	50S ribosomal protein L21	-2.10	More in SCs	8.63E-05
RpoA	RNA polymerase subunit alpha	-2.09	More in SCs	4.24E-03
RspA	30S ribosomal protein S1	-2.85	More in SCs	5.90E-04
RpsC	30S ribosomal protein S3	-3.33	More in SCs	4.50E-05
RpsD	30S ribosomal protein S4	-2.52	More in SCs	2.41E-03
RpsI	30S ribosomal protein S9	-2.45	More in SCs	1.39E-03
RpsJ	30S ribosomal protein S10	-3.99	More in SCs	3.81E-05
RpsK	30S ribosomal protein S11	-2.18	More in SCs	3.48E-02
RpsM	30S ribosomal protein S13	-2.65	More in SCs	2.80E-04
RpsS	30S ribosomal protein S19	-2.95	More in SCs	3.90E-02
SerS	Serine--tRNA ligase	-2.57	More in SCs	3.00E-04
Ssb	ssDNA binding protein	-2.33	More in SCs	2.88E-05
Tuf	Elongation factor	-3.13	More in SCs	2.89E-03
TypA	GTPase	-5.12	More in SCs	7.73E-05
TyrS	Tyrosine--tRNA ligase	-2.37	More in SCs	2.44E-03
YebC	Transcriptional regulator	-2.66	More in SCs	1.43E-02
YifE	Macrodomain ori organisation protein	-2.84	More in SCs	9.27E-03

Type III secreted effector proteins were found in the secretomes of both strains, which mirrored previously published data (Table 3.5) (Paulin *et al.*, 2007).

The differentiating factor was protein abundance. The enrichment of T3SS-1 effector proteins (8/10 detected) in the secretome of ST4/74 vs SCSA50 has not previously been reported. Similarly, decreased secretion of T3SS-1 effectors by host-restricted *Salmonella* serovar Paratyphi A has previously been shown by mass spectrometry profiling (Elhadad *et al.*, 2016). Additionally, western blot analysis has shown lower expression of SipD by *S.* Typhi compared to *S.* Typhimurium (Johnson *et al.*, 2018b), as the data presented here demonstrated for SCSA50 compared to ST4/74.

A protein which was expected to be in higher abundance in the secretome of ST4/74 was translocon effector SipC but despite western blotting evidence (Paulin *et al.*, 2007), the fold change in this experiment was calculated as 1.29 and therefore, classed as similar in both strain datasets. Juxtaposed with SipC, the fold changes calculated for the guanine nucleotide exchange factor SopE and E3 ligase SopA were 7.60 and 3.88, respectively, signifying that these proteins were highly enriched in the secretome of ST4/74. Since the gene encoding SopE is absent from the SCSA50 genome and the SCSA50 SopA is truncated, the high fold changes were unsurprising although it also suggests that the data analysis performed should have removed proteins absent in the secretomes of both strains.

The major function of most T3SS-1 effectors enriched in the ST4/74 secretome is to enhance bacterial cellular invasion and promote inflammation. SipA induces interleukin-8 (IL-8) and NF- κB expression (Figueiredo *et al.*, 2009; Marijke Keestra *et al.*, 2011), SipB activates Caspase-1 to induce apoptosis and the expression of IL-1B and IL-18 (Hersh *et al.*, 1999), SopA targets host ubiquitin ligases to stimulate the inflammatory interferon response (Kamanova *et al.*, 2016), SopE activates Rho-family GTPases Rac1 and Cdc42 to stimulate inflammation via Caspase-1

(Hoffmann *et al.*, 2010), and SopE2 induces IL-8 production in cells in concert with flagellin, (Huang *et al.*, 2004). SptP is an exception and inhibits pro-inflammatory pathways and actin remodelling activated by other effectors (Fu and Galán, 1999; Lin *et al.*, 2003) and SopF inhibits autophagy to promote intracellular bacterial replication (Xu *et al.*, 2019).

This data could be important in the context of *Salmonella* pathogenesis since comparative *in vivo* and *in vitro* experiments have demonstrated that *S.* Choleraesuis infections exert less IL-8 secretion and neutrophil recruitment than *S.* Typhimurium infections (Skjolaas *et al.*, 2006; Paulin *et al.*, 2007).

Another exciting finding from the mass spectrometry dataset was the enrichment of proteins that are either translocated by both secretion systems or only by T3SS-2 in the secretome of SCSA50 versus ST4/74. These effectors function to dampen pro-inflammatory NF- κB signalling (PipA) (Sun *et al.*, 2016), support the SCV (PipB, SteC, SifA and SopD2) (Knodler *et al.*, 2002; Poh *et al.*, 2008; D'Costa *et al.*, 2015; Zhao *et al.*, 2015), promote extraintestinal dissemination (SpvC) (Gopinath *et al.*, 2019) or have little defined function (SteB) (Geddes *et al.*, 2005).

The relative abundance of phosphothreonine lyase SpvC is a fascinating finding since the effector has a role in downregulating the inflammatory response in the intestines which was linked by a murine colitis model infection to increased systemic dissemination of *S.* Typhimurium (Haneda *et al.*, 2012) despite streptomycin pre-treatment (Barthel *et al.*, 2003).

SlrP, in contrast, is an E3 ubiquitin ligase which promotes cell death (Bernal-Bayard and Ramos-Morales, 2009). The enrichment of proteins that support the intracellular lifestyle of *Salmonella* in the secretome of SCSA50 was surprising. As previously shown, the *in vitro* conditions to induce T3SS-2 secretion are significantly different (Figure 3.2 and Figure 3.5) and translocation of T3SS-2 proteins was therefore unexpected.

PipB has been shown to be detectable by western blotting at very low levels under T3SS-1 conditions (Knodler *et al.*, 2002), suggesting that the sensitivity of mass spectrometry was essential for extensive secretome profiling. Addiitonally, one of the major transcriptional activators of T3SS-1, HilD, also positively regulates the two-component regulatory system SsrA-SsrB which controls T3SS-2 activity (Garmendia *et al.*, 2003).

The only effector protein enriched in the secretome of *S.* Paratyphi A versus *S.* Typhimurium was T3SS-1 or -2 effector SopD (Elhadad *et al.*, 2016), which has multiple roles during infection including aiding invasion and *in vivo* intracellular survival (Jiang *et al.*, 2004; Bakowski *et al.*, 2007) but this effector was not detected by either ST4/74 or SCSA50 in this experiment.

In addition to new questions about whether this data impacts the pathogenesis of these serovars, it also suggests that ST4/74 and SCSA50 gene expression could be differentially regulated.

High sensitivity proteomics is a valuable tool for defining the repertoire of proteins present in samples and for the identification of novel candidate effector proteins, especially when paired with mutant strains impaired in type III secretion (Niemann *et al.*, 2011; Cheng *et al.*, 2017). In this experiment, eight uncharacterised proteins were detected by mass spectromet–y - SCA50_0346, SCA50_0392, SCA50_2891, STM474_2071, STM474_2074, STM474_3876, YdgH and YgaM. These proteins were annotated as putative or hypothetical proteins by BLASTP and several contain domains of unknown function (DUF). Validation of the secretion and translocation of these uncharacterised proteins using T3SS null mutants is required.

Non-T3SS associated virulence factors were also present in the dataset such as the T1SS adhesin SiiE, the major component of type 1 fimbriae FimA, protease Ecotin, and various proteins related to intracellular survival (KatE, KatG, SodB, SodCI and PagC). Several of these virulence factors are cytoplasmic or periplasmic bacterial proteins that have previously been

identified in the *S.* Typhimurium secretome (Niemann *et al.*, 2011; Cheng *et al.*, 2017). PagC, a virulence-associated membrane protein, promotes the biogenesis of outer membrane vesicles (OMVs) (Kitagawa *et al.*, 2010) and its secretion could suggest that proteins within OMVs were detected.

Several proteins involved in metabolic pathways such as amino acid biosynthesis and breakdown, sugar metabolism, fatty acid biosynthesis and anaerobic metabolism were identified along with protein folding chaperones, elongation factors, and components of transcription and translational processes were also present. Whilst these proteins may be present in the secretome as a result of unavoidable minimal cell lysis, they could also be carried within outer membrane vesicles as previously published (Bai *et al.,* 2014) or be present on the bacterial cell surface.

With the Log_2 fold change for protein FlgK set as 0 during downstream analysis, differential enrichment of flagellar proteins was not expected. Since secreted components FlgL (fold change 6.49) and FliD (fold change -3.46) were enriched in either strain, independent validation of the dataset by western blotting is required.

3.4 Final conclusions

Label free quantitative LC-MS/MS proteomics revealed that host-adapted *SCS*A50 secretes less T3SS-1 associated effector proteins (Table 3.5) than generalist *ST*4/74 despite high sequence identity (Table 3.5) and a consistent rate of growth between the strains (Figure 3.3). In addition to reduced secretion of T3SS-1 effectors, the enriched secretion of effectors translocated by T3SS-2 by SCSA50 was identified.

Since T3SS-1 is required for the colonisation of the swine host by both *S.* Choleraesuis and *S.* Typhimurium (Lichtensteiger and Vimr, 2003; Carnell *et al.*, 2007), the characterised secretomes of ST4/74 and SCSA50 could give a greater insight into the pathogenesis of both strains *in vivo*. To fully understand the biological relevance of the differential protein secretion by the

two strains validation of individual proteins of interest by western blotting using the characterised *in vitro* conditions is needed.

Bioinformatic analysis of the published genome sequences (Richardson *et al.*, 2011) identified key differences between the strains including truncations and pseudogenes in effector encoding genes informed on the expected secretomes of both strains but also revealed high sequence similarity of encoded proteins. Since the characterised secretomes of ST4/74 and SCSA50 were profoundly different (Table 3.5), the high level of shared identity between the strains raises important questions underlying the regulation of the transcription, translation, and physical secretion of effectors by each serovar.

Additionally, the lack of shared SPI-16 nucleotide sequence identity between ST4/74 and SCSA50 is another observation worthy of further research. The small pathogenicity island encodes proteins related to O-antigen glucosylation which is important for bacterial persistence in a murine model of infection (Bogomolnaya *et al.*, 2008). Modification of the surface O-antigen has recently been linked to *S.* Paratyphi A inflammation and cell death pathways (Mylona *et al.*, 2020), and would therefore be an interesting candidate for future *S.* Choleraesuis versus *S.* Typhimurium host-adaptation research.

Another exciting prospect is the validation of the secretion and potential translocation of the uncharacterised proteins into host cells to determine whether any are novel candidate effector proteins by tagging the proteins of interest as other studies have (Geddes *et al.*, 2005; Niemann *et al.*, 2011).

It is well-described that gene expression in several bacterial species varies dramatically under different environmental conditions such as oxygen, temperature, and stress (Cho *et al.*, 2009; Mandlik *et al.*, 2011; Kröger *et al.*, 2012, 2013). ST4/74 and SCSA50 strains used could differ in response to environmental conditions *in vitro* versus how they respond to conditions *in*

vivo. The data suggests a great need to profile how ST4/74 and SCSA50 respond to host cell stimuli.

Collectively these results revealed large differences in the secretion of proteins by host-generalist ST4/74 and host-adapted SCSA50 using highly sensitive mass spectrometry, which is a novel comparison.

Chapter 4 Validation of the ST4/74 and SCSA50 secretomes

4.1 Introduction

In the animal host, the manifestation of salmonellosis is the most striking difference between *S.* Typhimurium and *S.* Choleraesuis infections. As representative strains, the pathology of ST4/74 and SCSA50 have been well-characterised in the porcine host (Bolton *et al.*, 1999; Paulin *et al.*, 2007).

ST4/74 causes enteric disease in pigs – the animals suffer from self-limiting diarrhoea, inflammation localised to the ileum and colon with enhanced influx of neutrophils to the gut lumen and upregulation of cytokines TNF-α, IL-8 and IL-18 (Paulin *et al.*, 2007). SCSA50, in contrast, does not commonly cause diarrhoea but disseminates systemically in the porcine host causing bacteraemia, enlargement of the liver and spleen and a high mortality rate (Paulin *et al.*, 2007). Despite these major differences in disease outcome, both *S.* Typhimurium and *S.* Choleraesuis require the major *Salmonella* virulence factor, the type III secretion system (T3SS), for colonisation *in vivo* (Lichtensteiger and Vimr, 2003; Carnell *et al.*, 2007; Chaudhuri *et al.*, 2013).

The secretion of proteins in a T3SS-dependent manner is evidently necessary for the virulence of both pathogens, and was the motivation behind the quantitative proteomic approach to profile the secretome of each strain in chapter 3 (Table 3.5). The strains were cultured under conditions akin to the intestinal lumen to induce T3SS-1, one of two T3SSs encoded by *S. enterica* strains, and label-free quantitative proteomics applied to the proteins secreted.

The most remarkable finding was the differential secretion of type III secreted effector proteins. ST4/74 secreted more T3SS-1 effectors than SCSA50 while SCSA50 secreted more effectors which are translocated by the second T3SS encoded by *S. enterica,* T3SS-2. Validation of the data is critical prior to extrapolating biological significance of the findings.

Label free quantitative proteomics has become an increasingly reliable method to quantify the abundance of proteins in a sample without the expense of isotope labelling and analysis software (Neilson *et al.*, 2011). Despite an experimental false discovery rate of less than 1% (described in materials and methods (chapter 2)), the comparative secretome data especially must be reproducible and be validated by western blotting with independent samples generated using the same method.

In addition to validating the differential secretion of proteins by ST4/74 and SCSA50, another key question is whether the secretion phenotype of these strains is representative of their wider respective serovar. Whilst these strains were originally field isolates from the bovine or porcine host, respectively, they have been used in laboratory practice since 1991 (ST4/74) (Jones *et al.*, 1991) and 1999 (SCSA50) (Bolton *et al.*, 1999), and therefore may not be representative of current clinically relevant strains.

Since the characterisation of the ST4/74 and SCSA50 secretomes has led to a greater knowledge of two strains which differ by host range, the data suggests that exploring the secretion profiles of other host-generalist, -restricted or -adapted serovars would lead to a greater insight into *S. enterica* zoonosis and biology.

The investigation, as detailed in results chapter one, also identified several hypothetical proteins which are of interest as candidate novel type III secreted effectors. Proteomic cataloguing of secreted effector proteins in an effort to identify novel substrates typically involves the employment of T3SS gene deletion mutants as a negative control (Niemann *et al.*, 2011; Vander Broek *et al.*, 2015; Cheng *et al.*, 2017). Without such controls, determining whether these proteins are T3SS substrates requires downstream validation with tagged proteins expressed in both wild type and mutant backgrounds.

Whilst the strains secreted over twenty T3SS effector proteins under standard laboratory conditions, the proteomic investigation neglected the

complexities of the host environment and the signals which trigger type III secretion such as iron, fatty acids, bile salts, and by-products of the microbiota (Nisco, 2018). The context of host cells could be important to both explore the biological relevance of *in vitro* type III secretion and understand what effect secretion has on invasiveness, considering evidence in other bacterial species that T3SS expression increases upon exposure to the host (Veenendaal *et al.*, 2007).

4.2 Chapter objectives

1. To validate proteins identified by mass spectrometry by immunoblotting using independent samples.
2. To interrogate a panel of *S.* Typhimurium and *S.* Choleraesuis strains to determine if differential T3SS secretion is serovar-specific.
3. To profile the expression of T3SS-1 secretion by a collection of *Salmonella enterica* serovars which differ by host range.
4. To investigate the sequences of hypothetical candidate effector proteins and whether they are secreted in a T3SS-dependent manner.
5. To compare the invasion of a porcine epithelial cell line by ST4/74 and SCSA50 under T3SS-1 inducing conditions.

4.3 Results and discussion

4.3.1 Detection of secreted type III effectors by western blotting

For validation of the differential secretion of proteins by ST4/74 and SCSA50 (Table 3.5), secreted and cell-associated proteins were probed by western blot using murine monoclonal antibodies raised against *S.* Dublin strain SD2229 proteins SipA, SipC, SopB and SopE2 and *Escherichia coli* cytosolic DNA chaperone DnaK.

These proteins were chosen to validate the biological relevance of the dataset, the differences in T3SS-1 effector protein secretion, and because of the availability of antibodies. DnaK was included both as a lysis control (if

secreted it indicates some bacterial cell lysis) and as a loading control for cell-associated proteins.

To generate samples in an independent experiment, the strains were cultured under the previously described T3SS-1 inducing conditions. Proteins secreted were precipitated and quantified while cell-associated proteins were extracted from 1 ml of culture. The cell-associated and secreted proteins (1 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting (Figure 4.1).

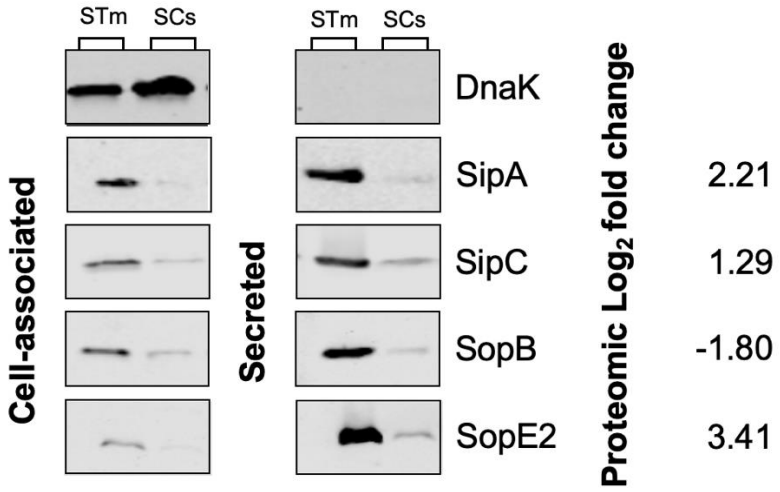


Figure 4.1 | The production and secretion of *Salmonella enterica* T3SS-1 proteins by ST4/74 (STm) and SCSA50 (SCs) during induction of T3SS-1.

Cell-associated proteins represent the proteins produced but not secreted during culture; secreted proteins are released into the supernatant.

DnaK was included as a loading (cell-associated) or lysis (secreted) control.

The proteomic Log_2 fold change represents the transformed ratio calculated from quantitative mass spectrometry data. Using 2 and -2 as cut-offs for proteins either more abundant in the secretome of ST4/74 (Log_2 fold change of > 2) or SCSA50 (Log_2 fold change of < -2). SipA and SopE2 were considered more abundant in the secretome of ST4/74 while SipC and SopB were classed as similarly secreted (Table 3.5).

Measurably different levels of all effector proteins were discovered both in the secreted and cell-associated fractions of four T3SS effector proteins (Figure 4.1). The above probed effector proteins – SipA, SipC, SopB and SopE2 – were identified with confidence in the proteomic dataset (Table 3.5). Western blotting validated the differential secretion of T3SS-1 effectors SipA and SopE2 by ST4/74 and SCSA50 uncovered using quantitative proteomics.

Whilst downstream statistical analysis of the proteomic database classed SipC and SopB as proteins which were secreted in similar amounts by both strains, independent western blotting validation with specific antibodies suggested that both proteins are also present in larger quantities in the ST4/74 secretome (Figure 4.1). As a technique to detect unique peptides and thus parent proteins, proteomics is highly sensitive but can be subject to bias or strict cut-offs during analysis.

Western blotting confirmed that cytosolic DNA chaperone DnaK was not secreted by either strain, demonstrating that differential T3SS effector secretion was not due to bacterial cell lysis. Interestingly, DnaK was identified as a differentially secreted protein by proteomics – it was more abundant in the secretome of SCSA50 (Table 3.5). This may have been a result of the sensitivity of mass spectrometry or variation between cultures prepared on different days.

Probing the cell-associated proteins of ST4/74 and SCSA50 also illustrated that SCSA50 also produced less of these effectors whilst the loading control DnaK was equally expressed by both strains (Figure 4.1). During the process of protein production, protection from degradation and finally translocation from the bacterial cytoplasm to the extracellular environment in an ATP-dependent manner (Kubori and Galán, 2002; Yu *et al.*, 2010), SCSA50 could differ from ST4/74 during regulation at either the transcriptional, post-transcriptional or translational level.

The proteins probed in Figure 4.1 were chosen because of their importance within the proteomic dataset and because of antibody availability but it must be noted that several other proteins were deserving of validation. T3SS-2 secreted effectors which were more abundant in the secretome of SCSA50 for example, would be of particular importance to future investigations.

The SipC western blot emulated the data generated by Paulin and colleagues who identified the reduced secretion of the effector by two *S.* Choleraesuis strains relative to three *S.* Typhimurium strains (Paulin *et al.*, 2007). The data, however, contrasts with that of a second study which compared the cellular proteome of *S.* Typhimurium strain LT2 and *S.* Choleraesuis strain SC-B67 cultured in RPMI cell culture medium (Huang *et al.*, 2016). Huang and colleagues concluded that SC-B67 produced more T3SS-1 effectors than LT2, but only validated their dataset using quantitative PCR rather than by western blotting. Without independent western blotting, confidence in proteomic data is reduced (Handler *et al.*, 2018). Moreover, the comparison of LT2, a laboratory-adapted strain of serovar Typhimurium which is avirulent in mice (Chaudhuri *et al.*, 2009), and SC-B67, a strain of serovar Choleraesuis isolated from a human sepsis patient (Chiu *et al.*, 2004), may be a less relevant comparison.

Like SCSA50, human-restricted typhoidal serovar Paratyphi A is similarly considered a low-secretor compared to *S.* Typhimurium (Elhadad *et al.*, 2016) and *E. coli* O157:H7 strain Sakai is a low-secretor compared to other *E. coli* strains (Roe *et al.*, 2003). These examples strengthen the theory that secreting relatively less effector proteins does not infer a less virulent pathogen and raises more questions about bacterial virulence strategies.

4.3.2 Are ST4/74 and SCSA50 representative of their respective serovars?

While ST4/74 is often used as a representative strain of *Salmonella* Typhimurium sequence type 19 (ST19), a major cause of gastrointestinal zoonotic disease worldwide (Pulford *et al.*, 2021), variation in virulence and

host-adaptation within *S.* Typhimurium is well-documented (Branchu *et al.*, 2018; Kirkwood *et al.*, 2021).

To determine whether the secretion phenotypes of ST4/74 and SCSA50 are representative of their respective serovars, a panel of *S.* Typhimurium and *S.* Choleraesuis strains were cultured under T3SS-1 inducing conditions. Strains were chosen which had been characterised in *in vivo* models of infection, been isolated recently in clinical studies or were present in *S. enterica* reference libraries.

Secreted and cell-associated proteins were generated and processed as previously described with an equal amount of secreted and equal volume of cell-associated protein separated by SDS-PAGE for western blotting. To compare the data with that of Paulin and colleagues (Paulin *et al.*, 2007), the production and secretion of SipC was probed (Figure 4.2).

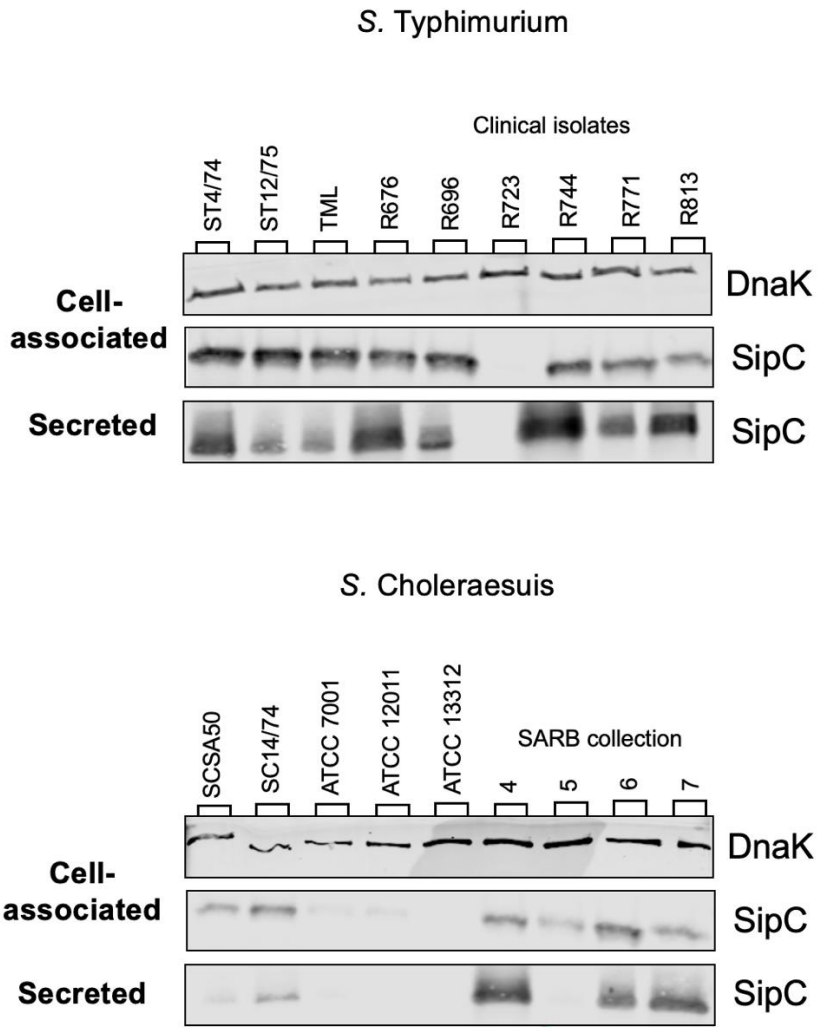


Figure 4.2 | Secretion of SipC by a panel of *S.* Typhimurium and *S.* Choleraesuis strains.

A panel of *S.* Typhimurium and *S.* Choleraesuis strains were cultured under T3SS-1 inducing conditions. Secreted and cell-associated proteins were isolated after the culture and probed for with antibodies raised against effector protein SipC and loading control DnaK.

Paulin and colleagues compared the secretion of SipC by *S.* Typhimurium strains ST4/74, ST12/75 and TML with *S.* Choleraesuis strains SCSA50 and SC14/74, and demonstrated that *S.* Choleraesuis strains secreted relatively less SipC (Paulin *et al.*, 2007). In Figure 4.2, the same strains were used and the data mirrored that of the 2007 study. With few exceptions, therefore, the production and secretion of effector SipC of ST4/74 and SCSA50 were representative of their wider respective serovars (Figure 4.2).

The panel of strains included for a wider survey of SipC secretion in *Salmonella* Typhimurium included ST12/75 and TML, two strains which are virulent in animal models of infection (Giannella *et al.*, 1973; Watson *et al.*, 1998). In addition to these *in vivo* tested strains, clinical isolates collected from cattle lymph nodes (R723, R744, R771) or faeces (R676, R696, R813) (Gragg *et al.*, 2013) were included to represent clinically-relevant strains of *S.* Typhimurium. It was surprising that R723 did not appear to produce or secrete SipC despite typical expression of loading control DnaK.

In addition to SCSA50, *S.* Choleraesuis strains probed include SC14/74, American Tissue Culture Collection (ATCC) strains 7001, 12011 and 13312, and *Salmonella* reference B (SARB) collection strains 4 to 7 (Boyd *et al.*, 1993). Whilst the virulence of SC14/74 has been published (Bolton *et al.*, 1999; Watson *et al.*, 2000), the interaction of ATCC strains and SARB collection strains with either cells or an animal model has not been investigated.

The above data illustrated that T3SS secretion can be a phenotype shared by a serovar, suggesting that exploring secretion by a range of serovars that differ by host range could give a greater insight into whether T3SS expression is interlinked with host-adaptation.

4.3.3 Secretion of SipC by host-generalist, host-restricted and host-adapted *S. enterica* serovars

To assess the secretion of T3SS-1 effector SipC by a range of *S. enterica* serovars, twenty-one strains were chosen to represent host-generalist

serovars Typhimurium, Agona, Derby, Enteritidis, Heidelberg, Infantis, Montevideo and Saintpaul; host-restricted serovars Abortusovis, Gallinarum and Typhisuis; and host-adapted serovars Choleraesuis and Dublin.

Most strains were part of the SARB collection and had previously been profiled at the genome level (Porwollik *et al.*, 2004, 2005; Torpdahl and Ahrens, 2004; Richardson *et al.*, 2011; Achtman *et al.*, 2013; Deng *et al.*, 2014; Sévellec *et al.*, 2019) but, with the exception of ST4/74 and SCSA50, no study has interrogated the expression of T3SS secreted virulence factors by these strains.

The strains were cultured under T3SS-1 conditions and secreted and cell-associated proteins extracted as previously described before western blotting was conducted (Figure 4.3).

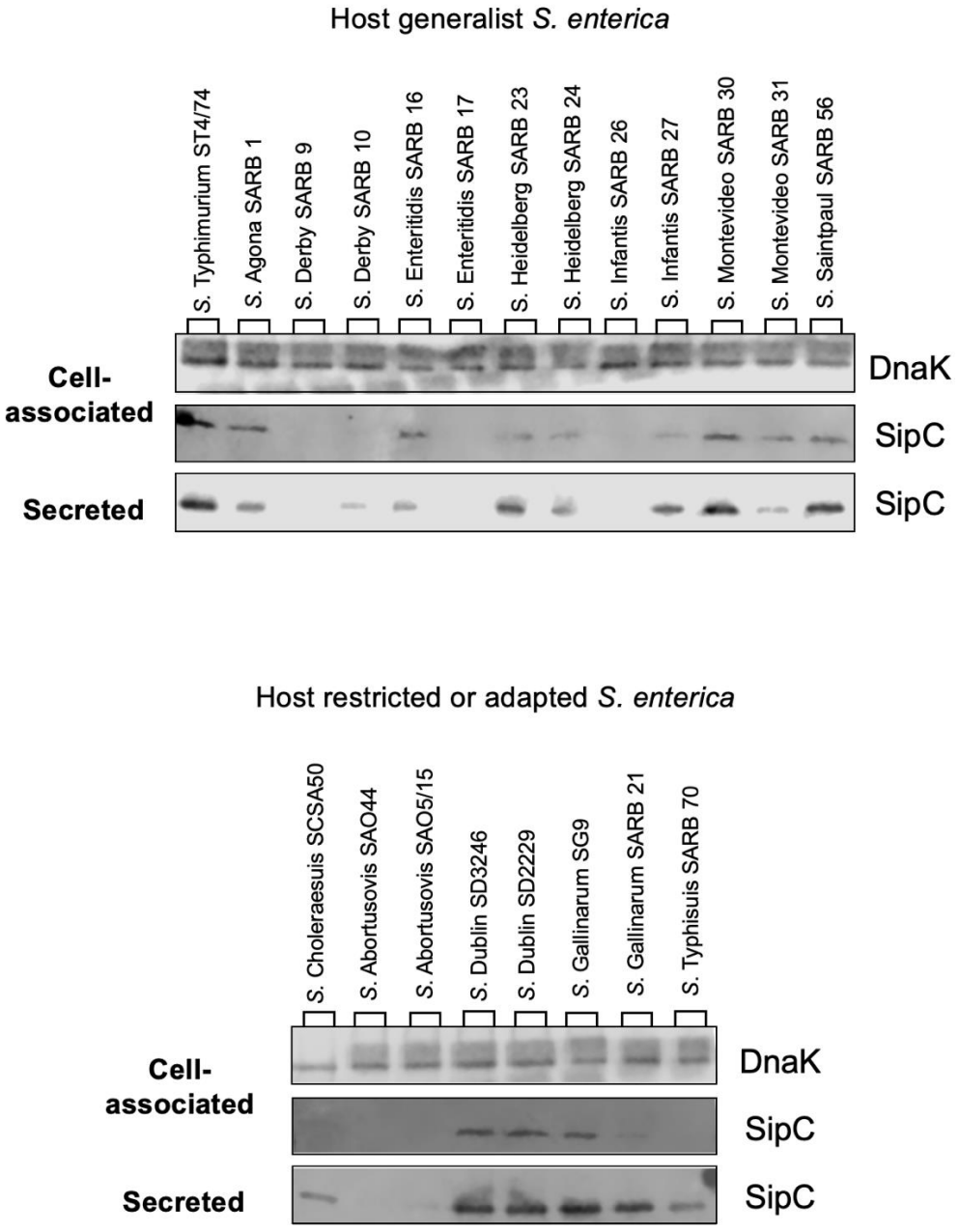


Figure 4.3 | Secretion of SipC by a panel of host-generalist, host-restricted and host-adapted *S. enterica* serovars.

A panel of host-generalist and host-adapted strains were cultured under T3SS-1 inducing conditions. Secreted and cell-associated proteins were isolated after the culture and probed for with antibodies raised against effector protein SipC and loading control DnaK.

The sheer diversity of *S. enterica* strains is demonstrated in Figure 4.3.

Probing for the secretion of SipC by serovars considered host-generalists illustrated great diversity, especially within serovars. With the exception of Heidelberg, each serovar represented by two strains showcased differential production and secretion of SipC which could be attributed to the isolation source and general diversity within a serovar. *S.* Enteritidis SARB 17, for example, is considered genetically divergent from common clinically isolated strains (Deng *et al.*, 2014) which suggests that a strain of more clinical relevance could be included to represent *S.* Enteritidis in future studies. There was also variation between the strains which represented serovar Derby, Infantis and Montevideo.

Between strains of host adapted serovars, however, production and secretion of SipC did not vary (Figure 4.3).

The virulence of the strains of host-restricted serovar Abortusovis have been investigated *in vivo* in ovine and bovine models of disease (Uzzau *et al.*, 2001; Paulin *et al.*, 2002) and defined as a systemic host-restricted serovar which invades the ovine intestine poorly despite its restriction to the sheep host (Uzzau *et al.*, 2001). Poor colonisation could correlate with reduced T3SS-1 expression.

S. Gallinarum is also host-restricted and causes enteric and systemic disease in poultry (Chadfield *et al.*, 2003) but, in contrast with *S.* Abortusovis, both *S.* Gallinarum strains secreted an equivalent amount of SipC as ST4/74 (Figure 4.3). Host-adapted *S.* Dublin also produced and secreted more SipC than *S.* Choleraesuis, equivalent to the level secreted by ST4/74. Swine restricted *S.* Typhisuis strain SARB 70 secreted similar amounts of SipC to swine-adapted SCSA50.

A notable conclusion from Figure 4.3 therefore is that reduced T3SS effector secretion was not universally linked to host-adaptation or host-restriction of *S. enterica.* There was, however, a pattern to be remarked upon in the small

collection of host-restricted and host-adapted serovars. *S.* Dublin and *S.* Gallinarum do cause enteritis in their respective hosts and both secreted as much SipC as ST4/74. SCSA50, *S.* Abortusovis and *S.* Typhisuis do not cause enteric symptoms and secreted less.

Overall, exploring the secretion phenotype of a wide array of serovars conveyed greater knowledge of serovars which are common zoonotic agents which cause concerning outbreaks of disease. *S.* Enteritidis, *S.* Typhimurium and *S.* Agona (EFSA and ECDC, 2018; ECDC, 2021) have caused recent multi-country outbreaks of foodborne salmonellosis in Europe while *S.* Dublin recently caused a multi-state outbreak in the US (CDC, 2019).

4.3.4 Investigating the sequences of hypothetical secreted proteins

Within the catalogue of proteins identified by quantitative proteomics (Table 3.5), several were categorised as hypothetical with no known function.

There were four of interest as potential secreted virulence factors – STM474_2071, STM474_2074, SCSA50_0346, and SCSA50_2891. The location of these candidates in the genomes of ST4/74 and SCSA50 was first probed using SnapGene viewer (http://snapgene.com/) and compared to the location of known T3SS effector proteins using BRIG (Alikhan *et al.*, 2011) (Figure 4.4).

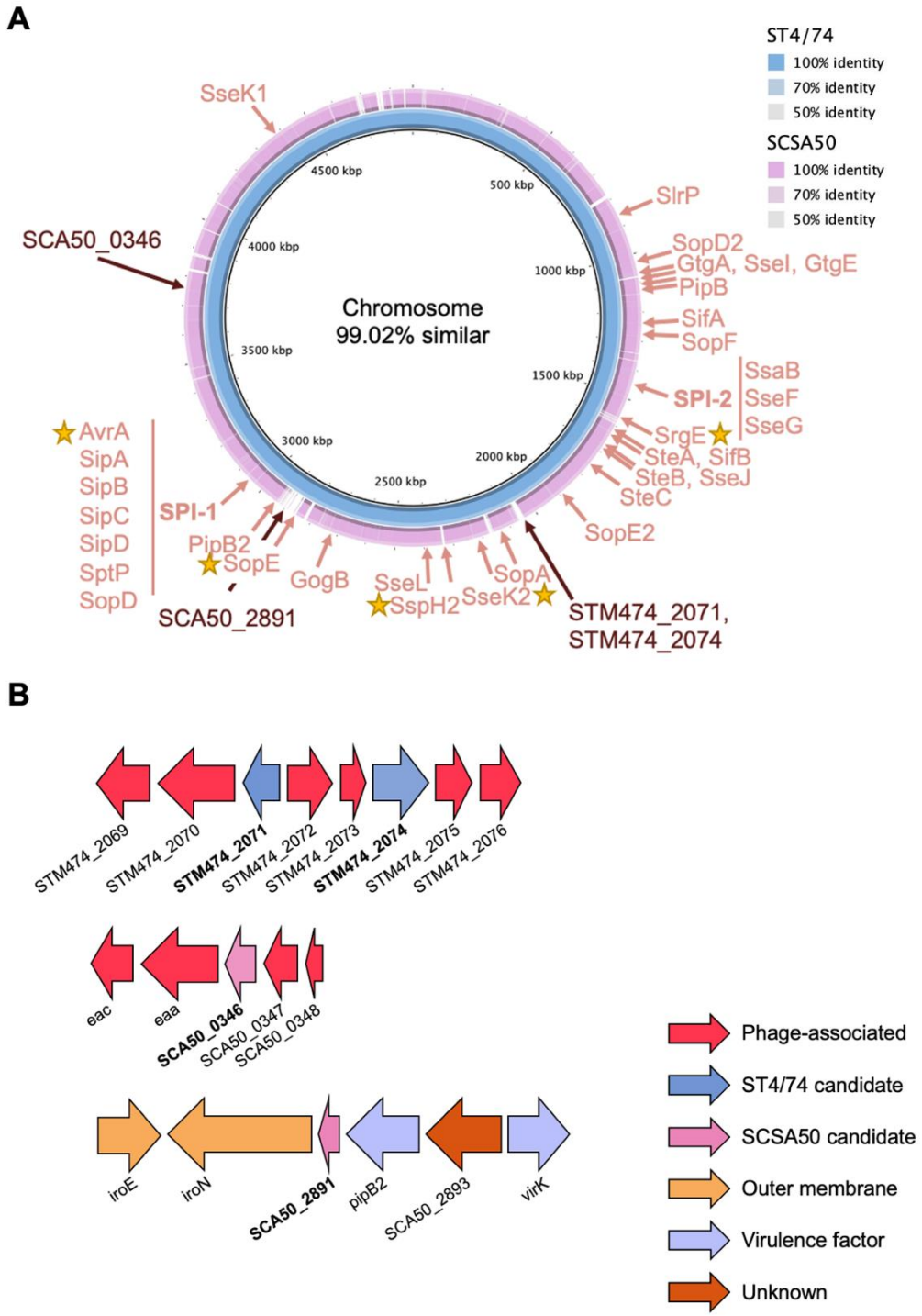


Figure 4.4 | Genomic context of the hypothetical proteins identified in the secretome of ST4/74 and SCSA50 under T3SS-1 inducing conditions

(A) Location of hypothetical proteins within the *Salmonella* genomes in the context of experimentally confirmed effectors of T3SS-1 and -2. The whole genome alignment was generated using BRIG. Stars represent proteins not present in the genome of SCSA50.

(B) Genetic location of hypothetical proteins which was determined using SnapGene viewer, the genome annotation and BLASTP.

STM474_2071 and STM474_2074 were encoded within the ST64B bacteriophage island, an island which also encodes T3SS-2 effector SseK3. SCA50_0346 was also surrounded by genes that are phage-associated while SCA50_2891 was encoded close to T3SS-2 effector PipB2.

Horizontal acquisition of virulence factors coded within the remnants of lysogenic bacteriophages is a common feature of the evolution of pathogenic bacteria (Conner *et al.*, 1998). For example, SPI-1 and -2, encoding T3SS-1 and -2, are both horizontally acquired in what is described as a “quantum leap” for bacterial pathogenesis (Groisman and Ochman, 1996).

Whilst the genomic context of hypothetical proteins is insufficient to assign them as hypothetical T3SS substrates, sequence analysis complemented highly sensitive mass spectrometry and added to the contention that these unknown proteins could be candidate effectors.

Further to investigating the location of the genes encoding the hypothetical proteins, the predicted amino acid translations were also probed for presence of eukaryotic linear motifs (ELMs) using the online ELM tool (http://elm.eu.org/) (Gouw *et al.*, 2018). Searching for the presence of eukaryotic linear motifs (ELMs) is a common method for the discovery of translocated virulence factors, as illustrated by a recent large genomic search for T4SS proteins conducted in *Legionella spp.* (Gomez-Valero *et al.*, 2019). The presence of motifs native to the host cell is often used to predict the function and cellular localisation of bacterial virulence factors (Gouw *et al.*, 2018).

If any hypothetical protein was discovered to be secreted and translocated in a T3SS-dependent manner, the presence of ELMs would direct future research (Table 4.1).

Table 4.1 | Eukaryotic linear motifs in hypothetical proteins.

The eukaryotic linear motifs identified by ELM software (Gouw et al., 2018) in hypothetical proteins STM474_2071, STM474_2074, SCA50_0346 and SCA50_2891.

Protein	Eukaryotic linear motif (ELM)	Examples
STM474_2074	14-3-3 binding phosphopeptide motif	Tir and EspF, T3SS effectors of *E. coli*
STM474_2071	Actin-binding motif	BimA, secreted virulence factor of *B. pseudomallei*
STM474_2071 STM474_2074	Caspase-3/7 cleavage site	SipA and SopA, T3SS effectors of *S. enterica*
STM474_2071 STM474_2074 SCA50_0346 SCA50_2891	MAPK docking motif	SpvC, T3SS effector of *S. enterica* and OspF, T3SS effector of *S. flexneri*
STM474_2071 SCA50_2891	MAPK phosphorylation site	IncA, T3SS effector of *C. trachomatis*
STM474_2071	Nuclear localisation signal	IntA, secreted virulence factor of *L. monocytogenes*
STM474_2071 SCA50_2891	PDZ domain ligand	Map, T3SS effector of *E. coli*
STM474_2071 SCA50_2891	SH3 ligand	EspF, T3SS effector of *E. coli*

All four hypothetical proteins contained ELMs also encoded by experimentally characterised T3SS effectors of different bacteria within the Enterobacteriace.

In addition to the genomic context of these proteins, surveying conservation of the encoding genes within *S. enterica* is a step towards determining their importance to bacterial virulence. To investigate conservation and relevance of the genes which encode the secreted hypothetical proteins, *S. enterica* serovars which pose risks to animal and human health were surveyed.

S. Typhimurium, Choleraesuis, Derby, Heidelberg, Infantis and Saintpaul are the serovars commonly isolated from infected pig farms (Rodríguez and Suárez, 2014) and were included as they pose real dangers to food safety and animal health. The nucleotide sequence of each hypothetical was surveyed using BLASTn and the data presented as a heat map which aided visualisation of % shared identity (Figure 4.6).

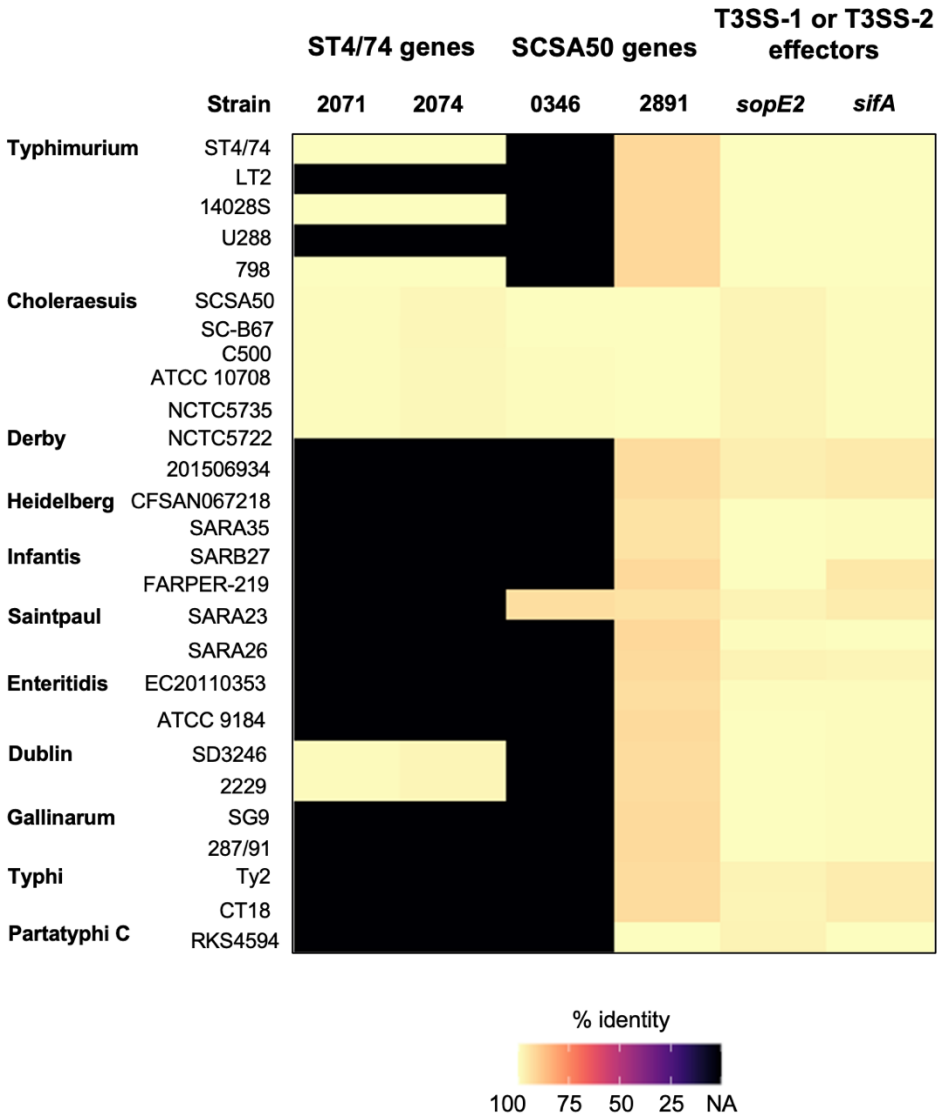


Figure 4.5 | Carriage of the genes encoding hypothetical proteins identified by proteomics.

tBLASTn was used to assess the carriage of each gene encoding hypothetical proteins STM474_2071, STM474_2074, SCA50_0346 and SCA50_2891 and experimentally characterised effectors SopE2 and SifA in genomes of *S. enterica* serovars important to public health. The % identity was calculated from either the genome of ST4/74 or SCSA50 and the data visualised using ggplot2.

The genes encoding the four hypothetical proteins were diversely carried across the twenty-seven genomes probed.

SCA50_2891 was conserved across all strains surveyed but interestingly a homologue was also present in the laboratory workhorse *E. coli* strain K12, annotated as a phage gene. Despite this, however, the gene may still have promise as a candidate – integration into regulation networks is key for the expression of horizontally-acquired virulence factors. The variable carriage of STM474_2071 and STM474_2074 could represent the variable carriage of the ST64B phage (Figure 4.6).

The historic research bias toward *Salmonella* Typhimurium has resulted in a wealth of large *in vitro* and *in vivo* screens such as transposon directed insertion site sequencing (TraDIS), proteomics, and RNA sequencing. One important study assigned ST4/74 genes to the intestinal colonisation of pigs, cattle, and chickens (Chaudhuri *et al.*, 2013). Whilst an insertion in STM474_2071 did not affect the fitness of *S.* Typhimurium in any livestock species tested, an insertion in STM474_2074 was not present within the mutant library. Interestingly SCA50_2891 is actually a pseudogene in ST4/74 but insertion in the intergenic region between *iroN* and *pipB2* had a significant fitness cost for *S.* Typhimurium colonisation of pigs, cattle, and chickens (Chaudhuri *et al.*, 2013).

4.3.5 Probing the secretion of candidate effectors

The most fundamental experimental investigation of candidate effector proteins is determining dependence on the T3SS for secretion into culture media (Vander Broek *et al.*, 2015). If secreted, translocation into host cells would confirm a hypothetical protein as a novel T3SS effector.

cyaA gene fusions are a common strategy used to confirm translocation of candidate *S. enterica* effector proteins into the host cytosol (Jones *et al.*, 1998; Wood *et al.*, 2000b; Geddes *et al.*, 2005; Niemann *et al.*, 2011) first used to investigate proteins secreted by *Yersinia* (Sory and Cornelis, 1994).

Effector-*cyaA* fusions are generated using the protein N-terminal region and the adenylate cyclase domain (*cyaA*) of the adenylate cyclase toxin of *Bordetella pertussis*. If translocated and delivered to the host cytosol, CyaA is activated by the host cell protein calmodulin and intracellular cyclic AMP (cAMP) can be consequently be measured (Young and Palmer, 2017).

To study the secretion and translocation of the aforementioned hypothetical proteins, *cyaA* fusions were planned and would comprise a Shine Dalgarno site, the first N-terminal 100 amino acids of the protein of interest, the *B. pertussis cyaA* domain, a 6xHis tag and stop codon. Translocation into host cells could be tested using the *cyaA* domain and secretion into LB broth tested using an anti-His antibody. The fusion would be ligated into vector pBluescript KS+, the same plasmid used by two *S. enterica* studies (Jones *et al.*, 1998; Wood *et al.*, 2000b).

Unfortunately despite attempted optimisation by PCR-Ligation-PCR (Ali and Steinkasserer, 1995), no constructs were generated. gBlock synthetic DNA (IDT) was ordered but due to time constraints the experiments were not completed.

If completed, the constructs were to be transformed into Enteropathogenic *E. coli* (EPEC) wild-type strain E2348/69 and gene deletion mutant E2348/69 Δ*escN* which has no functional T3SS (Garmendia *et al.*, 2004). EPEC strains would be used since *E. coli* encodes only a single T3SS, a feature also exploited by researchers studying *B. pseudomallei* secreted proteins (Whitlock *et al.*, 2008; Gong *et al.*, 2011; Vander Broek *et al.*, 2015) and there was no double T3SS-1 and T3SS-2 gene deletion mutant in the laboratory archive.

4.3.6 Invasion of a porcine jejunal cell line under T3SS-1 inducing conditions

It is important to remark that the *in vitro* culture conditions used to generate proteomic samples lack environmental cues such as the alkalinity, high

osmolarity, and short chain fatty acids of the intestinal lumen (Bajaj *et al.*, 1996).

In addition, contact with host cells has been demonstrated to increase the expression of type III secreted effectors by *E. coli* strains that readily secrete effectors into culture media (Beltrametti *et al.*, 1999; Wachter *et al.*, 1999). The complexity of T3SS-1 expression is a fascinating feature of *Salmonella* biology and demonstrates the limitations of studying bacteria cultured under one condition at a single timepoint. Both *in vitro* and *in vivo* conditions, however, do share the low oxygen concentration theorised to be an inducing signal for T3SS-1 of *S. enterica* (Bajaj *et al.*, 1995; Sturm *et al.*, 2011) and *Shigella flexneri* (Marteyn *et al.*, 2010). These extrinsic signals are sensed by a multitude of systems which control chemotaxis, quorum sensing, and, crucially, regulation of virulence factors.

T3SS-1 inducing conditions were thus used to prime bacteria for invasion of the porcine intestinal epithelial cell line IPEC-J2, investigating whether SCSA50 invasion would be attenuated compared to ST4/74 and a T3SS-1 mutant *in vitro* (multiplicity of infection (MOI) ~ 20) using a 30-minute gentamicin protection assay. Bacteria were brought into contact with the monolayer by centrifugation, allowed to invade for 30 minutes before the cells were overlain with fresh media containing the antibiotic gentamicin for a further 30 minutes before subsequent lysis and enumeration of intracellular bacteria on agar (Figure 4.7).

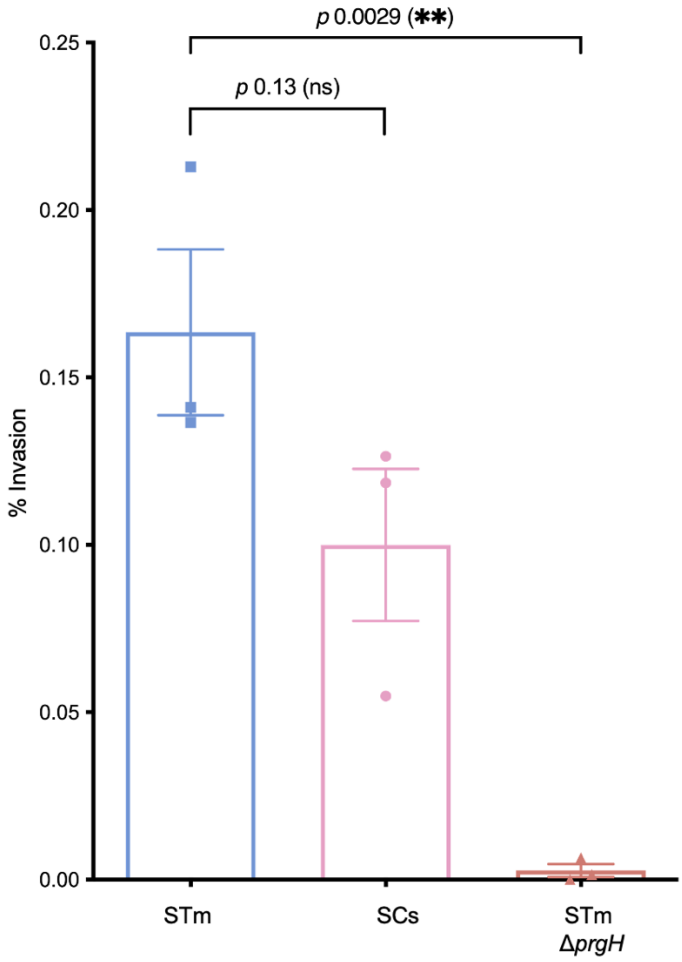


Figure 4.6 | Invasion of IPEC-J2 cells under T3SS-1 inducing conditions

1 hour invasion of IPEC-J2 cells was assessed after priming the bacteria with T3SS-1 inducing conditions using ST4/74 wild-type, SCSA50 wild-type, and ST4/74 Δ*prgH*. Invasion % was calculated as % of initial inoculum (quantified by retrospective plating of the bacteria).

ST4/74 was more invasive than SCSA50 after culture under T3SS-1 inducing conditions (Figure 4.7) but the data was not significantly different when a student's t test was used to compare % invasion. The T3SS-1 mutant, ST4/74 Δ*prgH,* however, did invade significantly less than wild-type ST4/74. Since gentamicin protection was performed after 30 minutes, these results represent *Salmonella* invasion, and little extracellular or intracellular replication.

The results illustrated that secreting relatively less T3SS effectors in culture does not significantly diminish invasion to the same level as a gene deletion mutant.

4.4 Final conclusions

Despite the high sensitivity of mass spectrometry (MS) as a technique, laboratory validation with appropriate controls is vital prior to extrapolating biological significance of findings. Since the secretomes of ST4/74 and SCSA50 were profiled using a label-free approach, potential variation between individual MS runs means that validation was especially fundamental.

Probing for a subset of T3SS-1 secreted effectors by western blotting illustrated the same finding revealed by quantitative proteomics – ST4/74 secretes more T3SS-1 effectors than SCSA50. Blotting also uncovered the differential production of T3SS-1 effectors by the strains despite equivalent amounts of DnaK (Figure 4.1). To unravel the mechanism underlying why ST4/74 produces and secretes more T3SS-1 than SCSA50, future investigation into regulation would give a greater mechanistic insight into these findings.

It is well-described that gene expression in several bacterial species varies dramatically under different environmental conditions such as oxygen, temperature, and stress (Cho *et al.*, 2009; Mandlik *et al.*, 2011; Kröger *et al.*, 2012, 2013). Assaying invasion of a porcine epithelial cell line suggested that

while there was a correlation between reduced secretion of T3SS-1 effectors in culture and reduced invasion, invasiveness was not significantly different between strains.

A meaningful future experiment could assess whether host cell contact influences the translocation of effectors secreted by either T3SS. The *cyaA* fusion approach, whilst it was not used for the hypothetical proteins investigated in this chapter, could be used to measure the differential translocation of effectors after contact with the host cell. Due to the secretion phenotypes and reduced invasion observed from the cultured bacteria, it would be anticipated SCSA50 to translocate less T3SS-1 effectors than ST4/74 and more effectors of T3SS-2.

As discussed previously, there is an abundance of different stimuli present for bacteria within host cells versus culture in broth (Bajaj *et al.*, 1996) and moreover, cell contact is a major stimulus for the translocation of effectors in *E. coli* (Beltrametti *et al.*, 1999; Wachter *et al.*, 1999).

The *S. enterica* proteome changes upon infection of cell types from different organs such as the gut epithelium, liver, and macrophage (Abshire and Neidhardt, 1993; Burns-Keliher *et al.*, 1998). Quantifying differences in the translocation of effectors between ST4/74 and SCSA50 within the context of host cells derived from different organs would therefore be a vital step toward speculating how our differential secretion data applies during a real infection.

The data in this chapter also demonstrated the diversity of *S. enterica* serovars considered host-generalist, host-restricted or host-adapted. Whilst ST4/74 and SCSA50 were determined to be representative of their respective serovars, reduced secretion of T3SS-1 effectors is not a universal trait of host-adapted or host-restricted serovars.

Chapter 5 Transcriptional regulation of type III secretion in ST4/74 and SCSA50

5.1 Introduction

Modulation of gene expression by complex regulatory networks is a crucial component of bacterial growth, survival and pathogenicity in ever-changing environmental conditions.

T3SSs are complex transmembrane needle structures that extend from the bacterial cytoplasm to the environment and secrete up to 10,000 amino acid residues per second (Singer *et al.*, 2012) in an ATP-dependent hierarchical manner (Akeda and Galán, 2004; Diepold and Wagner, 2014; Galán *et al.*, 2014). The energy required for expression of the T3SS needle apparatus and secretion of synthesised effector proteins slows bacterial growth and must therefore be tightly regulated (Saini *et al.*, 2010; Sturm *et al.*, 2011). The two *Salmonella* T3SSs are encoded on large horizontally acquired pathogenicity islands (SPI-1 and -2) which were integrated into structured regulatory networks by the bacterial chromosome to ensure their appropriate expression after their acquisition (Groisman and Ochman, 1996).

SPI-1 is a 40 kb chromosomal region which includes genes encoding the T3SS-1 apparatus, effectors, effector chaperones and transcriptional regulators *hilA, hilC, hilD,* and *invF.*

HilC, HilD and RtsA (which is encoded outwith SPI-1) regulate their own transcription and independently bind to and activate the central OmpR/ToxR family central regulator HilA in a coupled positive feedback loop (Saini *et al.*, 2010; Sturm *et al.*, 2011). Whilst the AraC/XylS-type DNA-binding transcriptional activators (HilC, HilD and RtsA) have a powerful effect on expression of *hilA* and therefore on T3SS-1 (Ellermeier *et al.,* 2005; Golubeva *et al.*, 2012), other regulators encoded outside of the pathogenicity island also exert transcriptional control over the system. A major example is HilE, a DNA-binding protein that negatively regulates the transcription of

T3SS-1 and is encoded downstream of SPI-1 (Baxter *et al.*, 2003; Grenz *et al.*, 2018). Once transcribed and translated, HilA activates the transcription of the SPI-1 gene clusters encoding the T3SS-1 needle and effector proteins (Ellermeier *et al.,* 2005).

SPI-2 is a 25 kb locus upstream of SPI-1 which includes genes encoding for T3SS-2 and its principal transcriptional activator SsrA-SsrB (Shea *et al.*, 1996; Cirillo *et al.*, 1998). SsrA-SsrB is a two-component regulatory system which itself is regulated by several factors encoded outside of SPI-2 including HilD, two-component systems PhoP-PhoQ and OmpR-EnvZ and nucleoid-associated protein Hha (Lee *et al.*, 2000; Garmendia *et al.*, 2003; Olekhnovich and Kadner, 2007; Bustamante *et al.*, 2008). SsrB, the response regulator of the two component SsrA-SsrB system, binds to the promoters of T3SS-2 associated genes to regulate their transcription (Walthers *et al.*, 2007) including those encoded outwith SPI-2 (Worley *et al.*, 2000). Transcriptional control of SPI-1 and SPI-2 is interconnected by several regulators (Figure 5.1).

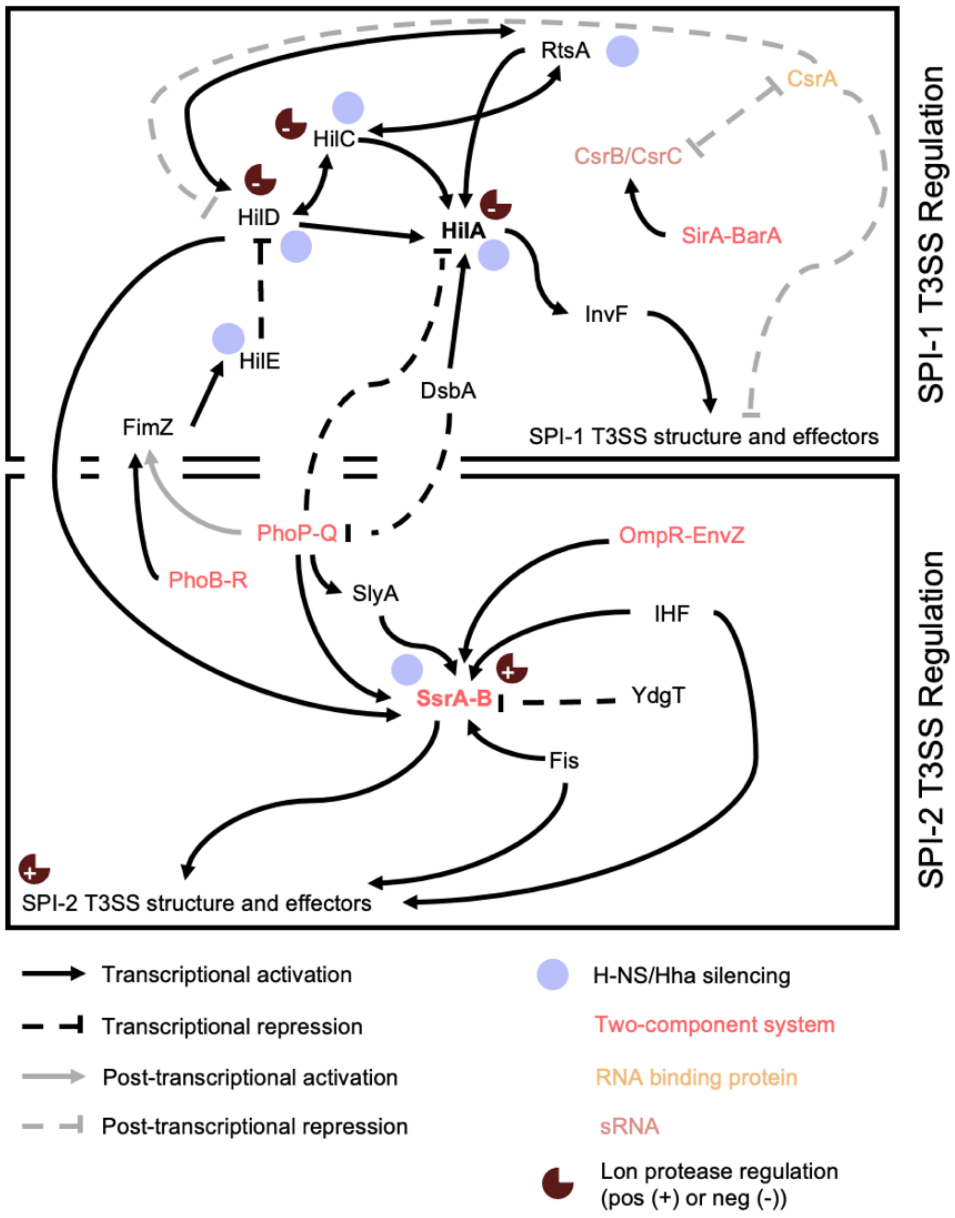


Figure 5.1 | Regulation of SPI-1 and SPI-2 T3SS of *Salmonella enterica* serovar Typhimurium

Regulation is a multi-layered process in *S. enterica* and takes place at the transcriptional, post-transcriptional and translational levels.

The complexity of the network of regulators controlling T3SS expression ensures that secretion systems are expressed in response to specific environmental cues such as osmolarity, pH, temperature and oxygen tension (Duong *et al.*, 2007; Mizusaki *et al.*, 2008; Gong *et al.*, 2009; Yu *et al.*, 2010; Kröger *et al.*, 2013; Steinmann and Dersch, 2013; Kim *et al.*, 2019).

Two-component systems OmpR-EnvZ and BarA-SirA are key signal transduction components of the regulatory network which sense and respond to environmental signals and affect central T3SS-1 regulator HilA (Bajaj *et al.*, 1996; Erhardt and Dersch, 2015; Lou *et al.*, 2019). Under inappropriate conditions for the expression of the T3SS, several transcriptional activators are subject to silencing by nucleoid-associated proteins H-NS and Hha which are global modulators of gene expression, and preferentially target horizontally-acquired genes with a lower GC-content (Navarre *et al.*, 2006; Dorman, 2007; Olekhnovich and Kadner, 2007).

In addition to transcriptional regulators, post-transcriptional regulation is critical for coordination of virulence and fitness in Gram-negative bacteria (Volk *et al.*, 2019). RNA binding proteins such as CsrA, Hfq, and ProQ have an important role in the control of T3SS regulators, structure and effectors post-transcriptionally (Westermann *et al.*, 2019) (Figure 5.1).

For example, binding of CsrA to mRNA target motifs both prevents translation of several T3SS effectors and regulators and affects the stability of the transcripts (Altier *et al.*, 2000; Martínez *et al.*, 2011; Vakulskas *et al.*, 2015; Potts *et al.*, 2019). In addition to CsrA binding, the transcripts of key transcriptional activator *hilD* are also acted upon by RNAses (RNase E and PNPase) and Hfq to control *hilD* and T3SS-1 expression (López-Garrido *et al.*, 2014).

Once translated, T3SS-associated proteins are then regulated by proteases which exert post-translational control on bacteria. Lon protease, as an

important example, negatively regulates central T3SS-1 regulator HilA (Takaya *et al.*, 2002) by degrading HilC and HilD (Takaya *et al.*, 2005).

S. enterica type III secretion is evidently tightly regulated, with multiple layers of control which could influence the differential secretion of proteins demonstrated by ST4/74 and SCSA50.

Several studies have dissected the differences in T3SS expression between *Salmonella* serovars and a pattern of lower transcriptional expression of SPI-1 genes in host-adapted or -restricted serovars versus host-generalist has been identified (Paulin *et al.*, 2007; Elhadad *et al.*, 2016; Johnson *et al.*, 2017). There is, however, mounting evidence that expression of genes within virulence networks varies within serovars and contributes to diverse disease presentations (Clark *et al.*, 2011).

The significantly different translation and secretion of effectors between ST4/74 and SCSA50 characterised in this thesis led to the hypothesis that there would be a significant difference in the transcription of genes encoding effectors and the transcriptional regulators that control them. This was investigated using RT-qPCR in an effort to identify a molecular mechanism at the transcriptional level controlling the two strains.

5.2 Chapter objectives

1. To investigate the transcription of the genes encoding T3SS secreted effector proteins.
2. To examine the transcription of effector protein chaperones by ST4/74 and SCSA50.
3. Survey the sequence identity shared between ST4/74 and SCSA50 in the T3SS regulatory gene network.
4. Identify differences in transcription of the regulators of T3SS-1 and -2 between ST4/74 and SCSA50.

5.3 Results and discussion

5.3.1 Experimental design and characterisation of internal control *rpoD*

Bacteria were cultured under T3SS-1 conditions as previously described. The number of bacteria used to extract RNA from was first optimised and contamination with genomic DNA troubleshooted.

RNA was thus extracted from ~3x10^8 bacterial cells during induction of T3SS-1 activity at two time points, first after overnight culture at 30 ˚C (baseline) and secondly after subculture at 37 ˚C (induced). This was repeated thrice to retain three independent biological replicates for each strain and at each timepoint.

Despite equivalent viable counts at these two phases of growth, expression levels of housekeeping genes could not be assumed equal between the two strains. Therefore, collecting RNA from two conditions and normalising transcripts separately was important. RNA quality was assessed by Nanodrop $A_{260/280}$ values, DNase treated and 500 ng aliquots stored at - 20 ºC prior to cDNA synthesis.

Highly sensitive reverse transcription-quantitative PCR (RT-qPCR) was then employed to assess expression of mRNA transcripts in ST4/74 and SCSA50 to inform on the transcriptional expression of the encoding genes.

The housekeeping gene chosen as an internal control was *rpoD*, an RNA polymerase subunit whose expression is not directly correlated with bacterial growth phase in several bacterial species (Navarre *et al.*, 2005; Botteldoorn *et al.*, 2006; Gal-Mor *et al.*, 2008; Cameron *et al.*, 2017). This gene was previously demonstrated as an appropriate internal control between two *S. enterica* serovars *S.* Typhimurium and *S.* Paratyphi A (Elhadad *et al.*, 2016) and as *rpoD* was found to be highly similar between ST4/74 and SCSA50 (99.5% shared nucleotide identity) it was chosen as the internal housekeeping control.

Prior to testing a range of cDNA concentrations, three primer concentrations were tested before the final concentration of 200 nM chosen (data not shown). Contamination of cDNA with genomic DNA (gDNA) was also assessed prior to RT-qPCR. For each replicate, cDNA was synthesised from RNA with or without reverse transcriptase (RT) and amplification of *rpoD* tested using conventional PCR (Figure 5.2).

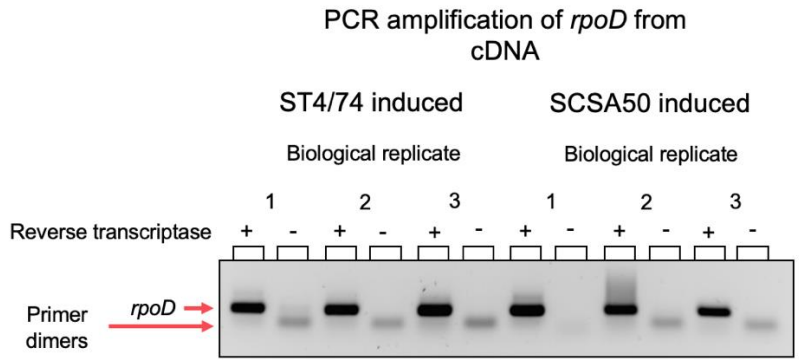


Figure 5.2 | PCR amplification of *rpoD* by cDNA synthesised with or without RT

cDNA was synthesised from RNA with reverse transcriptase (+ RT) or without (- RT). Conventional PCR amplification of *rpoD* was performed on all samples to assess whether there was genomic DNA contamination.

The agarose gel pictured in Figure 5.2 demonstrated that there was no significant gDNA contamination in any biological replicate. Additionally, all samples synthesised using reverse transcriptase yielded a strong product of the correct size (Figure 5.2). Whilst Figure 5.2 only illustrated the induced samples, the experiment was also repeated with baseline samples (data not shown).

There was, however, a faint band amplified from ST4/74 cDNA synthesised without RT (biological replicate 1). To control for any gDNA contamination which would influence the RT-qPCR data, the cDNA (-RT) for each strain was diluted 1:10, pooled together and included on every qPCR plate as an essential control.

RT-qPCR was then optimised for *rpoD* at the two time points discussed above with the average of three technical replicates used for each biological replicate (Figure 5.3).

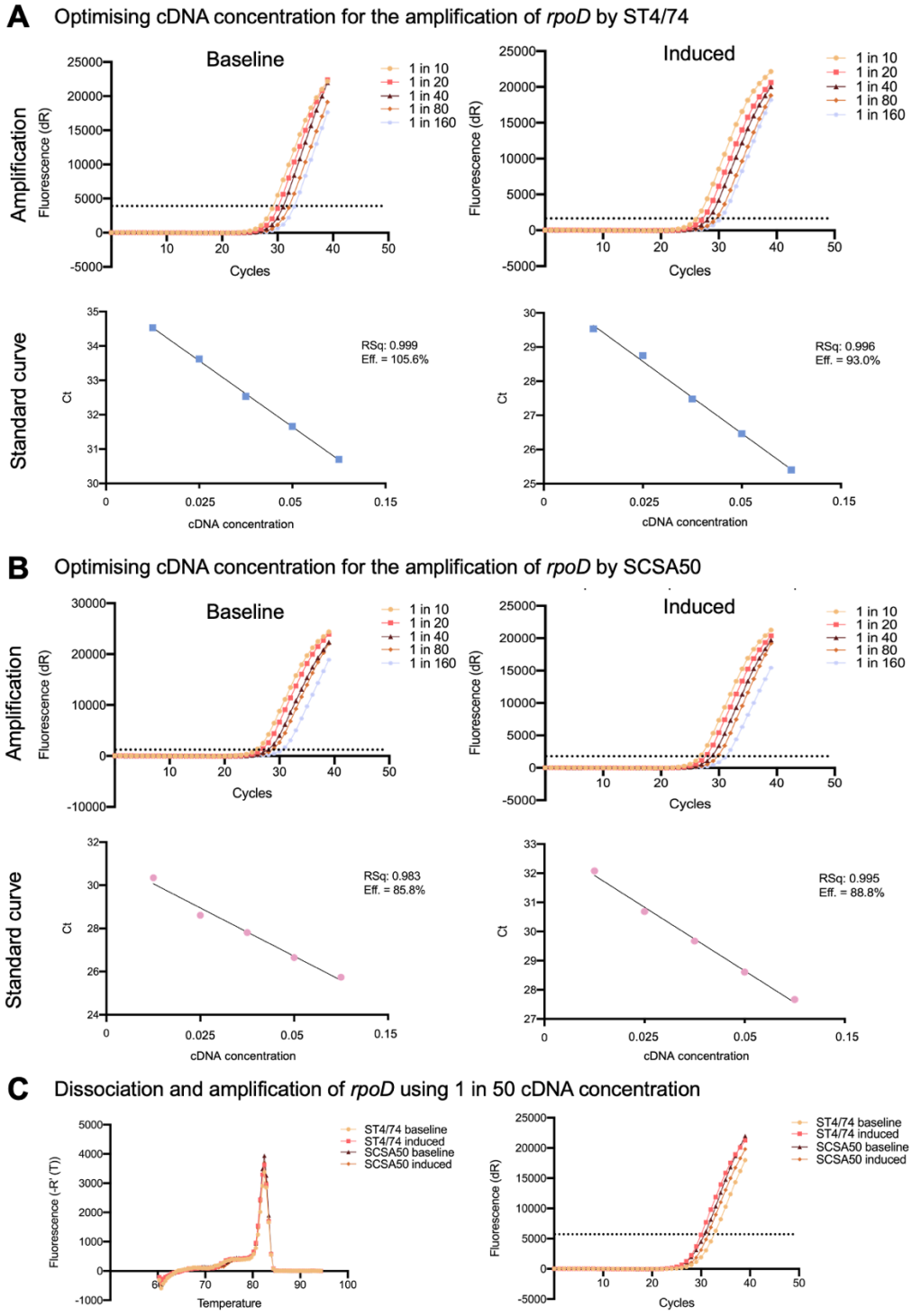


Figure *5.3* | Optimisation of the amplification of housekeeping gene *rpoD* by qPCR.

(A) Optimisation of appropriate cDNA concentration for amplification of ST4/74 *rpoD* under two conditions using serially diluted cDNA to generate standard curves.

(B) Optimisation of appropriate cDNA concentration for amplification of SCSA50 *rpoD* under two conditions using serially diluted cDNA to generate standard curves.

(C) The amplification of a single product using cDNA diluted 1:50 was proven by the single dissociation curve.

The data indicated that amplification of *rpoD* was efficient from either strain (Figure 5.3).

The optimisation of cDNA concentration was completed by serially diluting cDNA to obtain a standard curve fitted with a linear regression. The RSq value that measures how close the Ct values fit the regression line was above 0.98 for all four standard curves.

The Eff. (efficiency) value measures the efficiency of the reagents within the qPCR reaction. The cDNA generated from SCSA50 was slightly less efficient than optimal (90-110%), potentially due to differences in the annealing of primers but was still well within acceptable range. Despite designing primers in a region of *rpoD* which is conserved between ST4/74 and SCSA50, the primers could have potentially contributed to differences in PCR reaction efficiency.

For each of the biological replicates, amplification of *rpoD* resulted in a single product (Figure 5.3C, dissociation curve). Unfortunately, the observation was made retrospectively that the amplification of *rpoD* from these experimental samples was subject to a small level of variation (Figure 5.3C, amplification plots) and upon reflection, the best housekeeping gene would be at a constant level of expression between all samples and both strains.

Whilst it is true that housekeeping genes can vary within an experiment, this should be considered a caveat to comparing two different strains of *S. enterica* and taken into consideration when interpreting the qPCR dataset.

Because of this small level of variation between baseline and induced samples within strains, it was decided that the *rpoD* data would be used for data analysis using both the Pfaffl (Pfaffl, 2001) and $2^{-\Delta\Delta CT}$ (Livak and

Schmittgen, 2001) analysis. The latter does not require data from both baseline and induced samples.

5.3.2 Transcriptional expression of type III effector encoding genes

Within the profile of secreted proteins identified by quantitative mass spectrometry (Table 3.5), SifA, PipB and PipB2 were more abundant in the secretome of SCSA50 while SipC was more abundant in the secretome of ST4/74. The SipC data was confirmed by western blotting but due to a lack of antibodies against T3SS-2 effectors SifA, PipB and PipB2, these proteins were not validated.

Using RT-qPCR, the abundance of *sipC, sifA, pipB* and *pipB2* transcripts were hence investigated in ST4/74 and SCSA50. The same method was used to determine the final primer and cDNA concentration as *rpoD* (data not shown) before data analysis to explore transcript abundance (Figure 5.4). Fold changes represent the fold change from baseline to induced, normalised to *rpoD* (Pfaffl) (Figure 5.4A) or fold change from *rpoD* to gene of interest ($2^{-\Delta\Delta CT}$) (Figure 5.4C).

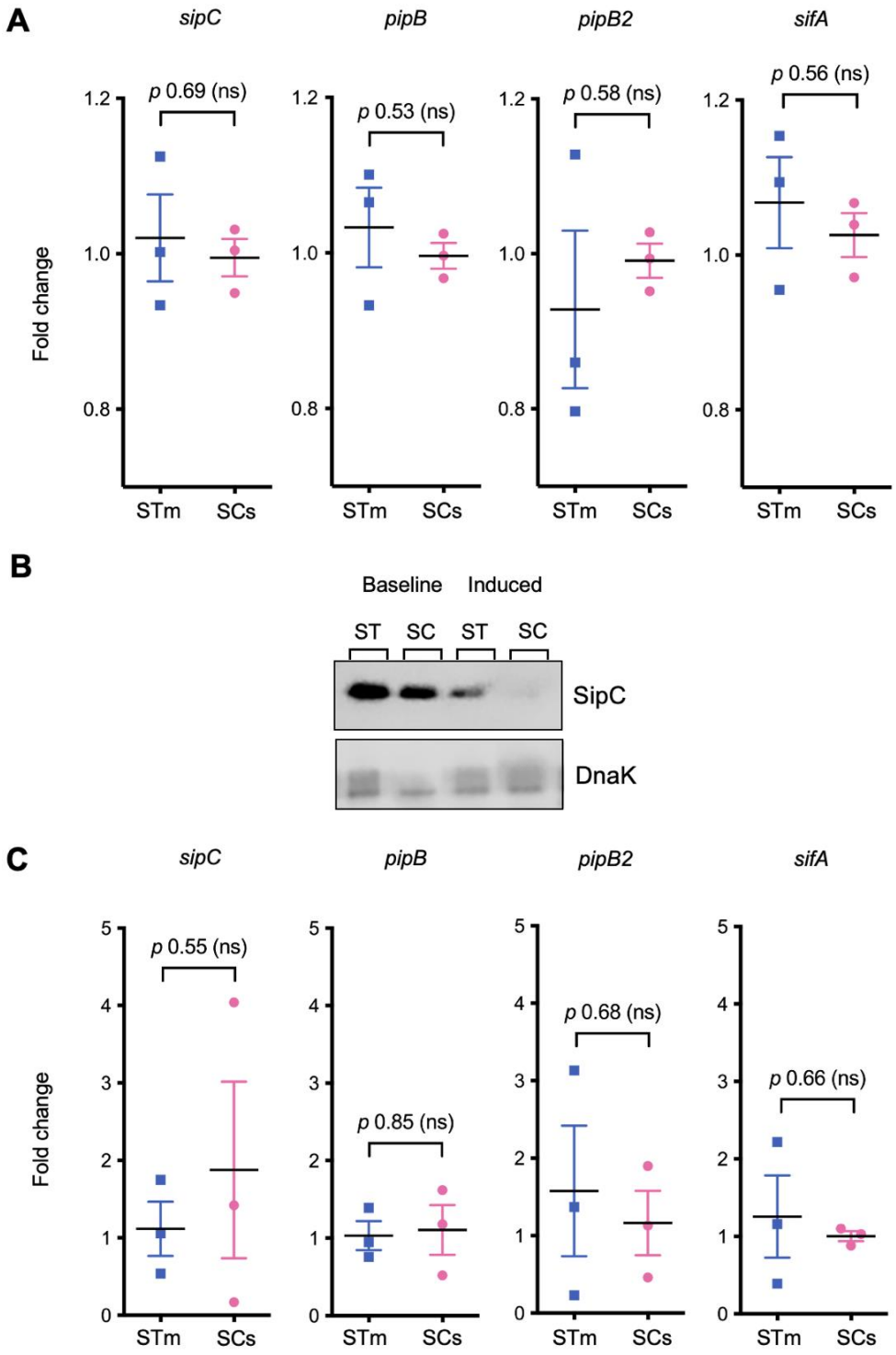


Figure 5.4 | Transcriptional expression of genes encoding effector proteins *sipC, pipB, pipB2* and *sifA* by ST4/74 (STm) and SCSA50 (SCs).

(A/C) Each data point represents one biological replicate (the average of 2-3 technical replicates). To calculate significant difference, unpaired two-tailed student's t tests were used.

(A) Fold change in mRNA abundance between baseline and induced *S. enterica* normalised to rpoD and analysed using the Pfaffl method.

(B) Abundance of cell-associated SipC protein in ST4/74 and SCSA50 after overnight culture (baseline) and four hours of T3SS-1 induction at 37 °C (induced) was tested by western blotting. DNA chaperone DnaK was included as a loading control.

(C) Fold change in transcript abundance relative to housekeeping gene rpoD, using data from only the induced samples analysed by $2^{-\Delta\Delta CT}$.

The data presented in Figure 5.4A demonstrate fold changes in transcription of genes between two different growth states, where protein production was dramatically different (Figure 5.4B). Separate normalisation was considered an important calculation since the same *rpoD* housekeeping data cannot be used to normalise both ST4/74 and SCSA50. Interestingly, there was no significant difference in fold change induction of transcription of all genes probed when a student's t test was employed (Figure 5.4A).

Probing for cell-associated SipC by immunoblotting (Figure 5.4B) demonstrated that, unlike the repression of *Yersinia* spp. T3SS below 37 ºC (Hoe and Goguen, 1993), overnight culture of ST4/74 and SCSA50 at 30 ºC resulted in increased secretion of effector SipC compared to induced cultures. This may explain why the average fold change in transcript abundance of *sipC* between baseline and induced was 1.02 for ST4/74 and 0.99 for SCSA50 (Figure 5.4A) which indicated no increase in transcript abundance by temperature shift.

The relative quantification strategy $2^{-\Delta\Delta CT}$ was then employed to analyse the expression of *sipC, pipB, pipB2* and *sifA* relative to *rpoD* in induced samples only (Figure 5.4C).

Similar to fold changes calculated using Pfaffl analysis, the data was subject to variation. Variation between biological replicates could be explained by the bistability of T3SS expression as previous studies have shown that only a small proportion of bacterial cells within a culture express the T3SS needle as its expression is not conducive for replication and growth (Hautefort *et al.*,

2003; Sturm *et al.*, 2011). This method was used by several studies (Elhadad *et al.*, 2016; Aviv *et al.*, 2019) to compare gene expression between two *S. enterica* serovars and, unlike Pfaffl data analysis Figure 5.4A), did not take the efficiency of the PCR amplification into consideration. The fold changes presented were calculated relative to the housekeeping gene *rpoD* of each strain. There were no significant differences in transcription of any of the genes between the strains under either Pfaffl or $2^{-\Delta\Delta CT}$ analysis. This was a noteworthy result since immunoblotting demonstrated a dramatic reduction in translated cell-associated SipC protein by induced SCSA50 relative to ST4/74 (Figure 5.4B).

The data implies that translation and secretion of these effector proteins may be controlled post-transcriptionally. Several studies have investigated post-transcriptional control of effector protein expression including control of *sipC* by DksA (Rice *et al.*, 2015) and *avrA* and *sopD2* by CsrA (Kerrinnes *et al.*, 2009; Holmqvist *et al.*, 2016). As a translational control protease, Lon represses T3SS-1 expression by degrading HilC and HilD (Takaya *et al.*, 2002, 2005) and promotes T3SS-2 expression (Song *et al.*, 2019).

Salmonella bacteria evidently use several layers of regulation to control the network of virulence genes to enable an appropriate and fast response to environmental conditions. This complexity implies that investigating mRNA transcripts is inappropriate for validating proteomic datasets.

5.3.3 Transcriptional expression of type III effector chaperones

In addition to effector proteins, the transcriptional expression of three important chaperone proteins was explored. Chaperones are small proteins that bind effector proteins in the bacterial cytosol and load them onto the T3SS sorting platform which regulates the hierarchical secretion of effectors (Lara-Tejero *et al.*, 2011).

Since most type III secreted effectors are dependent on chaperones for protein stability and secretion, differential expression of protein chaperones

was hypothesised to be a mechanism by which ST4/74 and SCSA50 secrete different levels of effectors after T3SS-1 induction (Figure 5.4B). The three genes encoding chaperone proteins probed were *invB, sicA* (both T3SS-1) and *srcA* (T3SS-2) (Figure 5.5).

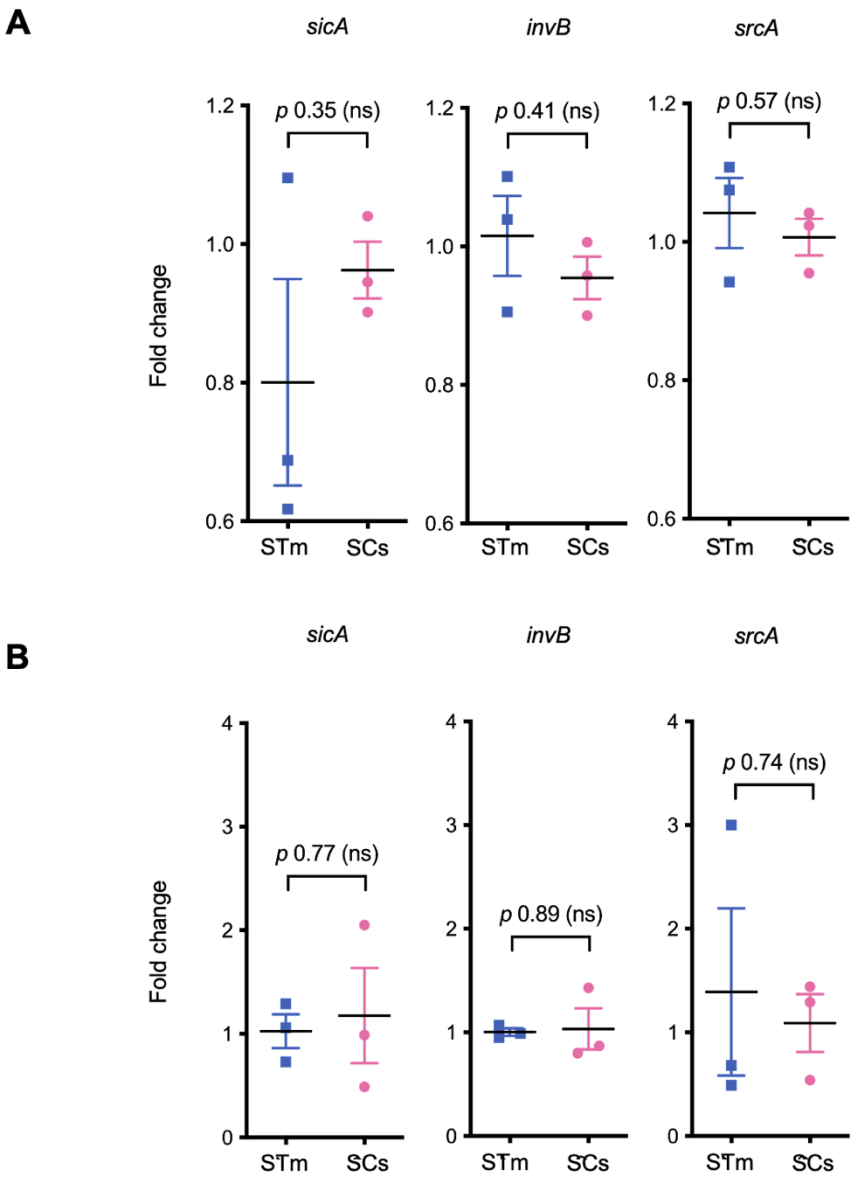


Figure 5.5 | Transcriptional expression of *S. enterica* encoded chaperone proteins *sicA, invB* and *srcA* by ST4/74 (STm) and SCSA50 (SCs).

(A/B) Each data point represents one biological replicate (the average of 2-3 technical replicates). To calculate significant difference, unpaired two-tailed student's t tests were used.

(A) Fold change in mRNA abundance between baseline and induced *S. enterica* normalised to rpoD and analysed using the Pfaffl method.

(B) Fold change in transcript abundance relative to housekeeping gene *rpoD*, using data from only the induced samples analysed by $2^{-\Delta\Delta CT}$.

There was no significant difference in transcription of chaperone proteins *invB, sicA,* and *srcA* when data was analysed either as a fold change between overnight and induced culture (Figure 5.5A) or as a fold change calculated relative to housekeeping gene *rpoD* (Figure 5.5B). There was, however, indication that there was more *sicA* induction in SCSA50 than ST4/74 but the data is subject to confounding variation (Figure 5.5A).

The SicA protein ensures SipB and SipC are translocated by T3SS-1, two proteins which were confirmed by western blot to be differentially secreted by ST4/74 and SCSA50. At the transcriptional level, however, there was little difference between ST4/74 and SCSA50 (Figure 5.5A). SicA is also involved in transcriptional activation of SPI-1 itself, complexed with InvF (Darwin and Miller, 2000, 2001; Tucker and Galán, 2000; Lahiri *et al.*, 2014). The data suggests probing chaperone protein expression using western blotting would lead to a greater understanding of protein expression by ST4/74 and SCSA50.

5.3.4 Shared sequence identity across the T3SS regulatory network

Most transcriptional activators contain a C-terminal helix-turn-helix DNA-binding domain which binds to the promoters of the genes they regulate (Aravind *et al.*, 2005). The sequence of either the DNA-binding domains of transcriptional activators or the promoters they bind are important for T3SS expression. The DNA and protein sequences of ST4/74 and SCSA50 regulators were examined using BLAST for differences in key regions, along with the experimentally defined promoter regions of the *hil* operon (Schechter and Lee, 2001; Olekhnovich and Kadner, 2002, 2006; Lim *et al.*, 2007) using BLASTn and Clustal Omega (Table 5.1).

Table 5.1 | Shared sequence identity of major SPI-1 and -2 regulators.

(A) NCBI BLASTn was used to calculate the % shared identity of promoters.

(B) NCBI BLASTn was used to calculate the % shared identity of genes and BLASTP used to calculate the % shared identity of proteins.

A

Regulator	% identity	Relevance
hilA	99.6%	SNP in the SCSA50 HilC and HilD binding region (A to G)
hilC	100%	
hilD	100%	
hilE	98.1%	Two SNPs in the SCSA50 *hilE* promoter in the FimZ binding region

B

Regulator	% DNA sequence identity	% amino acid identity
Major SPI-1 regulators		
HilA	100%	100%
HilC	99.66%	99.66%
HilD	99.68%	99.67%
HilE	97.54%	98.65%
RtsA	98.74%	99.31%
InvF	99.73%	97.99%
FimZ	99.14%	87.78%
Major SPI-2 regulators		
SlyA	99.09%	97.26%
OmpR	99.44%	97.91%
EnvZ	99.44%	99.78%
SsrA	99.18%	97.54%
SsrB	99.06%	99.53%
PhoP	99.11%	99.11%
PhoQ	99.11%	100%
YdgT	100%	76.31%

The bioinformatic analysis demonstrated several small differences in the DNA and protein sequences of the major SPI-1 and SPI-2 regulators (Table 5.1B) but none in experimentally defined DNA-binding regions (Narm *et al.*, 2020).

The promoters of genes within the *hil* operon were also probed (Table 5.1A). One single nucleotide polymorphism (SNP) was uncovered in the SCSA50 *hilA* promoter relative to ST4/74 at position -183 which was within the region that AraC/XylS-type transcriptional activators *hilC* and *hilD* (Schechter and Lee, 2001). Two SNPs were additionally uncovered in the SCSA50 *hilE* promoter within the *fimZ* binding region (Saini *et al.*, 2009). SNPs can influence binding of regulators to the promoter and therefore affect gene expression.

Testing whether expression of transcription or translation of HilA and HilE proteins differs between ST4/74 and SCSA50 will be essential for indirectly examining whether SNPs could influence the gene expression of these regulators.

5.3.5 Transcriptional expression of type III secretion regulators

To unravel regulatory differences upstream of effectors, the hyperinvasion locus (*hil*) operon, which is the major transcriptional regulator of T3SS-1, was studied. Predominantly encoded on SPI-1, the locus – consisting of master regulator *hilA*, positive regulators *hilC* and *hilD* and negative regulator *hilE* – was investigated for differences in transcription between ST4/74 and SCSA50 (Figure 5.6).

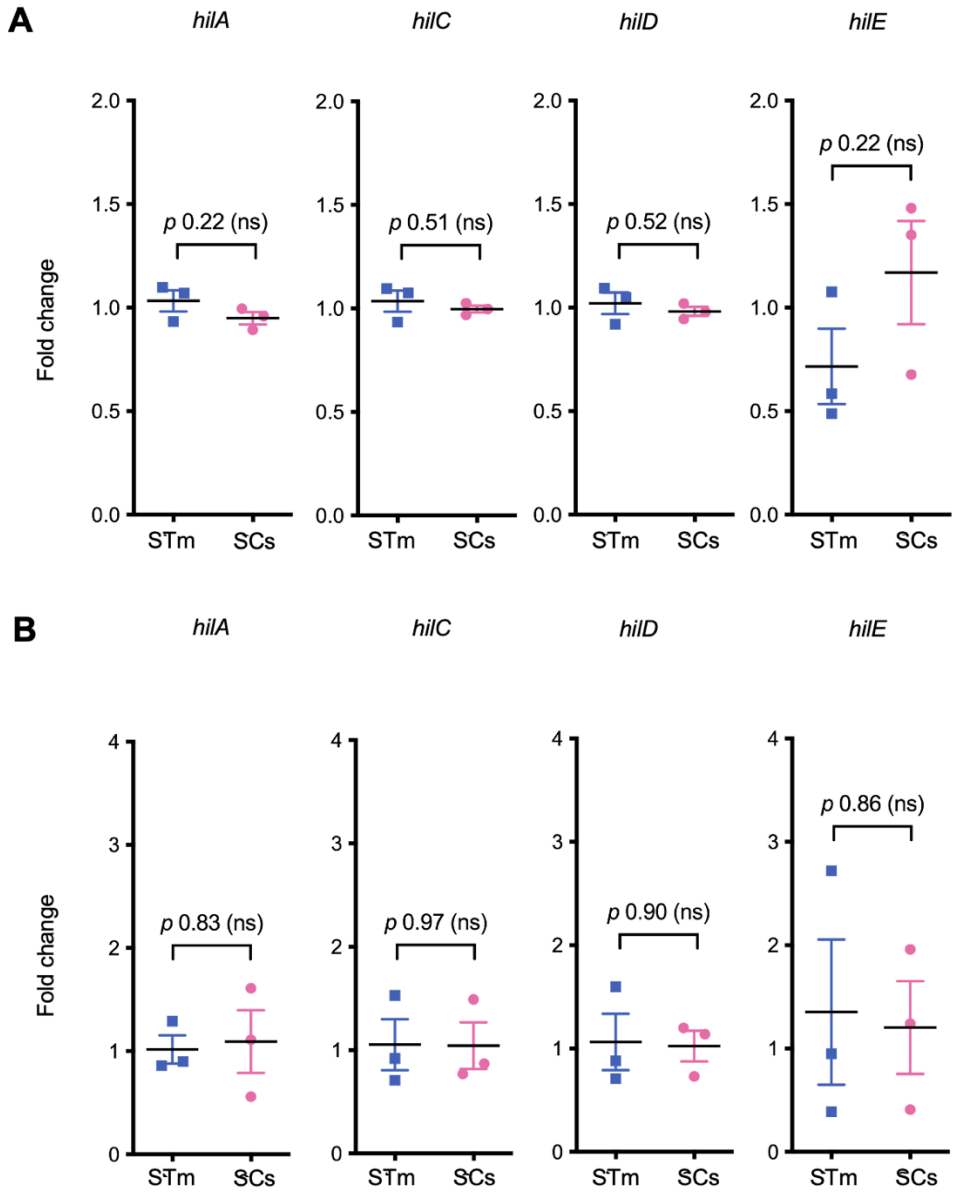


Figure 5.6 | Transcriptional expression of *S. enterica* encoded regulators *hilA, hilC, hilD* and *hilE* by ST4/74 (STm) and SCSA50 (SCs).

(A/B) Each data point represents one biological replicate cultured for four hours under T3SS-1 inducing conditions (the average of 2-3 technical replicates). To calculate significant difference, unpaired two-tailed student's t tests were used.

(A) Fold change in mRNA abundance between baseline and induced *S. enterica* normalised to *rpoD* and analysed using the Pfaffl method.

(*B*) Fold change in transcript abundance relative to housekeeping gene *rpoD*, using data from only the induced samples analysed by $2^{-\Delta\Delta CT}$.

The lack of significantly different transcriptional expression of the T3SS-1 regulatory network between ST4/74 and SCSA50 was surprising. There was a small biologically relevant trend toward higher transcription of *hilA, hilC* and *hilD* in ST4/74 and higher transcription of negative regulator *hilE* in SCSA50 but no statistically significant data (Figure 5.6A).

For *hilE,* a gene which is present at a low level in both strains under these conditions, the variation between biological replicates confounds any conclusions and suggests that more replicates may be required for definitive data interpretation (Figure 5.6A). Studying the expression of the operon relative to *rpoD* in induced cultures was also subject to variation and no trends could be extracted (Figure 5.6B).

Expression of transcriptional regulators is additionally dependent on the phase of bacterial growth and environmental conditions.

In vitro expression of *hilA* mRNA*,* the central transcriptional activator of the T3SS-1 structure and effectors, is optimal at early stationary phase where nutrients and oxygen become limited and conditions reflect the intestinal environment (Bajaj *et al.*, 1996; Bustamante *et al.*, 2008). Potentially, RNA was extracted after a degree of degradation had occurred and key differences in ST4/74 and SCSA50 were missed.

An interesting comparative study of HilD protein stability between *S.* Typhimurium and *S.* Typhi illustrated that in LB, the *S.* Typhimurium HilD had a half-life of 8 minutes (Johnson *et al.*, 2017). Determining whether RNA extracted from bacteria cultured to late logarithmic phase are the right conditions to assess regulator transcription would require additional time course culture experiments studying expression and potential degradation of mRNA.

A comparative study exploring regulation of T3SS-1 in *S.* Typhimurium and *S.* Paratyphi C which, similarly to SCSA50, secretes less T3SS-1 effectors than *S.* Typhimurium – found that *hilA* transcription influenced the differential

protein secretion (Elhadad *et al.*, 2016). Whilst the comparative qPCR approach presented in this thesis uncovered a trend toward higher transcription of *hilA* in ST4/74, this data suggests that, unlike *S.* Paratyphi C, there are other factors controlling the differential protein production and secretion between SCSA50 and ST4/74 such as post-transcriptional and translational factors. HilD, for example, which is the most important transcriptional activator of *hilA* and T3SS-1, is regulated post-transcriptionally by RNA-binding protein CsrA, translationally, and post-translationally (Olekhnovich and Kadner, 2007; Martínez *et al.*, 2011; Holmqvist *et al.*, 2016).

Secretion of T3SS-2 effectors is regulated by a multitude of factors including environmental conditions and multiple two-component systems. SsrAB, the two-component regulatory system, is critical for expression of T3SS-2 (Cirillo *et al.*, 1998) and is exclusively encoded by *S. enterica* (Gal-Mor *et al.*, 2011).

PhoPQ, conversely, is an ancestral two-component system in Gram-negative bacteria (Groisman, 2001) which senses extracellular magnesium concentration and binds to the *ssrB* promoter to post-transcriptionally regulate *ssrAB* (Bijlsma and Groisman, 2005) and additionally positively regulates the expression of T3SS-2 effector encoding gene *sseL* (Gal-Mor *et al.*, 2011). EnvZ-OmpR, is another two-component system that controls the expression of T3SS-2 via transcriptional activation of SsrAB (Lee, Detweiler and Falkow, 2000; Garmendia *et al.*, 2003).

The transcription of four important regulatory genes including *ssrB, phoP, ompR* and global gene silencer *hha* were assessed under the same T3SS-1 conditions which stimulated SCSA50 to secrete more T3SS-2 proteins than ST4/74 (Figure 5.7).

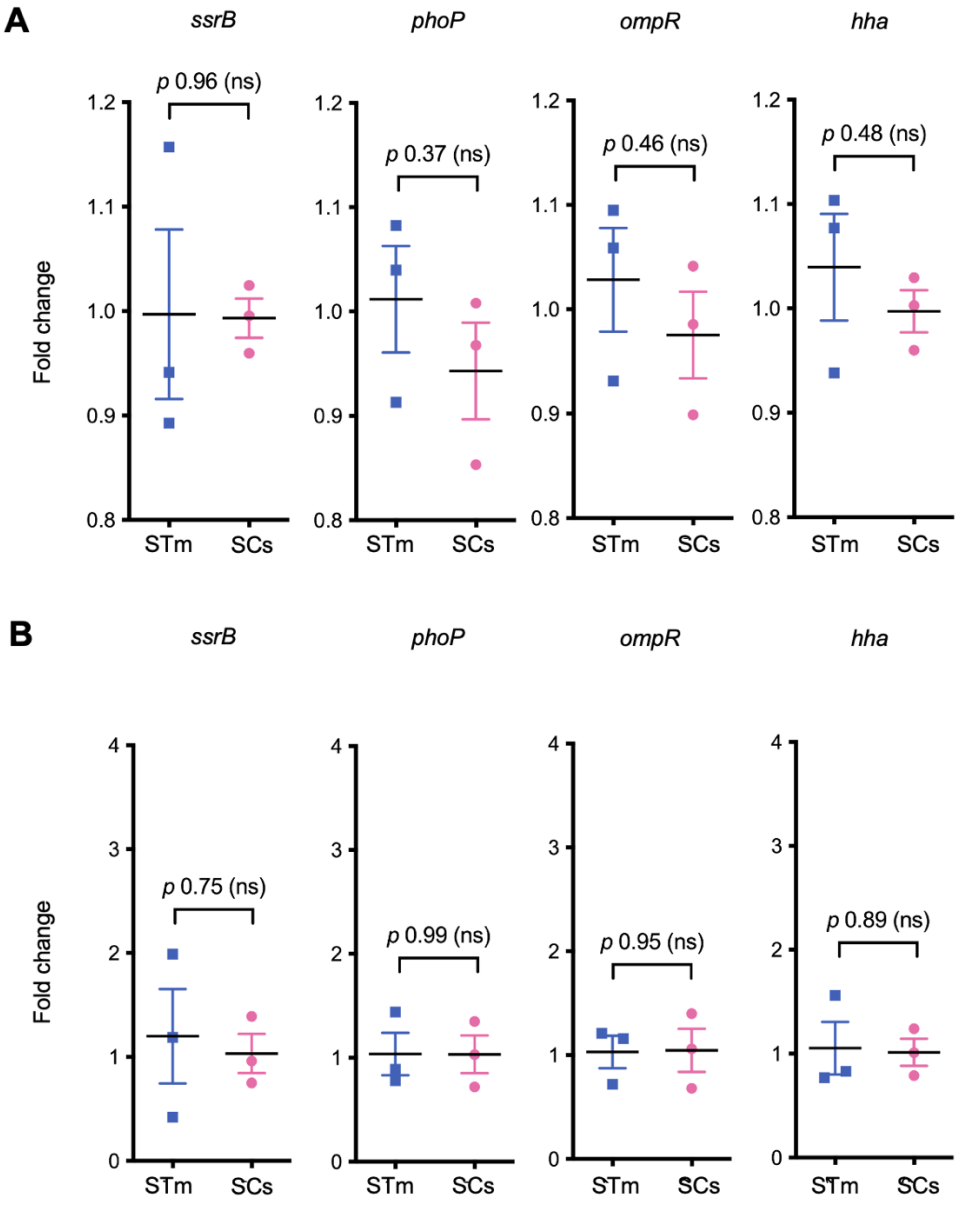


Figure 5.7 | Transcriptional expression of *S. enterica* encoded regulators *ssrB, phoP, ompR* and *hha* by ST4/74 (STm) and SCSA50 (SCs).

(A/B) Each data point represents one biological replicate cultured for four hours under T3SS-1 inducing conditions (the average of 2-3 technical replicates). To calculate significant difference, unpaired two-tailed student's t tests were used.

(A) Fold change in mRNA abundance between baseline and induced *S. enterica* normalised to *rpoD* and analysed using the Pfaffl method.

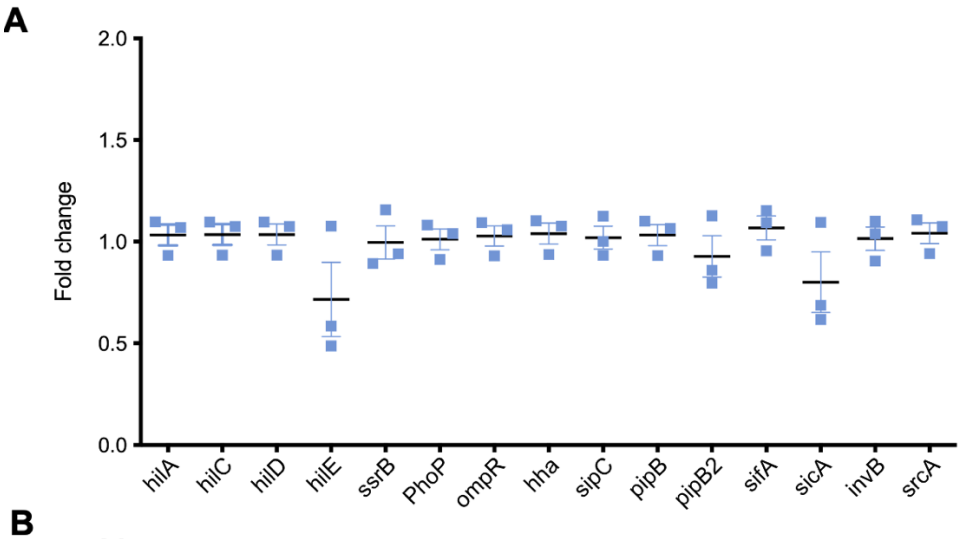
(B) Fold change in transcript abundance relative to housekeeping gene *rpoD*, using data from only the induced samples analysed by $2^{-\Delta\Delta CT}$.

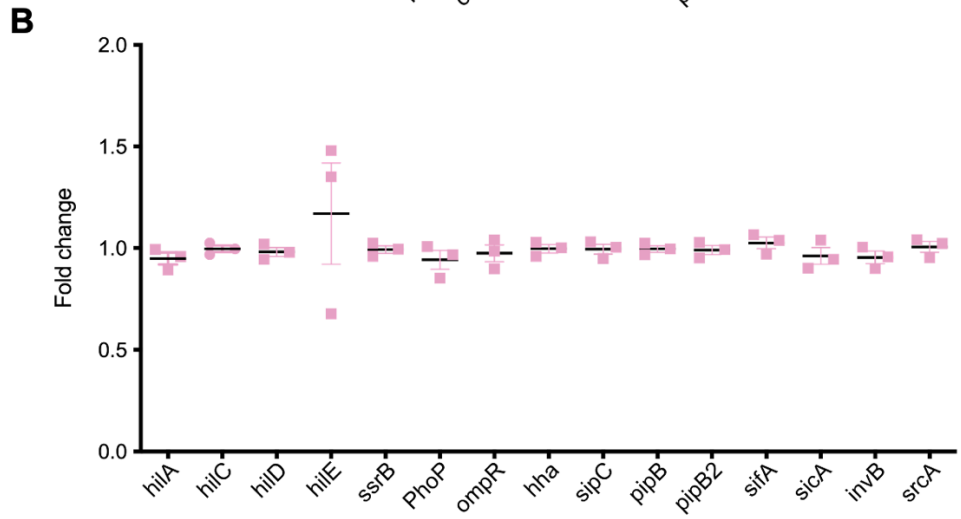
Despite differential secretion of T3SS-2 proteins between the strains, there was no statistically significant data comparing the expression of these regulators after either Pfaffl (Figure 5.7A) or $2^{-\Delta\Delta CT}$ (Figure 5.7B) analysis. In addition, biological variation between replicates, potentially due to the low level of expression of these genes, added further confounding elements when interpreting the data.

In vivo experiments in swine demonstrated that SsrB, the response regulator of SsrAB, is important for systemic salmonellosis but not gastrointestinal colonisation and disease (Boyen *et al.*, 2008). For virulence of *S. enterica* in murine models of disease and for intracellular survival in macrophages, both transcriptional activators of *ssrAB*, OmpR and PhoP, are additionally key (Chatfield *et al.*, 1991; Lee *et al.*, 2000; Groisman, 2001). This evidence suggests that whilst T3SS-2 translocated effector proteins were detected in the secretome of ST4/74 and SCSA50, the rich environmental conditions used here to replicate the intestinal lumen are possibly not optimal for the transcriptional expression of *ssrB, ompR and phoP.*

5.3.6 Summary data

The fold change data calculated for each gene, analysed using the Pfaffl method, was then plotted for each strain to investigate the effect of gene on fold change using the analysis of variance calculation (ANOVA) (Figure 5.8).





C

ANOVA					
Strain	**Factor**	**R squared**	***p* value**	***p* value summary**	**Significant?**
ST4/74	Gene	0.3995	0.2016	ns	No
SCSA50	Gene	0.2120	0.8620	ns	No

Figure 5.8 | Summary of fold change expression of genes screened

The fold changes calculated using the Pfaffl method were plotted for each strain to assess whether there was up-regulation of any genes during T3SS-1 induction (hour four).

(A) ST4/74 fold changes between overnight culture (baseline) and subculture (induced)

(B) SCSA50 fold changes between overnight culture (baseline) and subculture (induced)

(C) A one-way analysis of variance (ANOVA) was used to determine the effect of the gene on fold change.

The gene probed had no significant effect on the data (Figure 5.8C). The plot did, however, uncover a fascinating pattern. For both strains the transcription of most genes was not changed by T3SS-1 induction apart from *hilE.* Whilst variation is a factor, ST4/74 transcribed less *hilE* than other T3SS-1 regulators while SCSA50 transcribed more. This must be further investigated with more repeats.

Thus, within this series of RT-qPCR experiments to unravel differences between ST4/74 and SCSA50 at the transcriptional level, no mechanisms have been identified that could unequivocally explain the differences demonstrated by mass spectrometry. It is possible, but not experimentally confirmed, that post-transcriptional control distinguishes the two strains.

5.4 Final conclusions

The successful pathogenesis of *Salmonella* is dependent on the ability of the pathogen to coordinate sensing and responding appropriately to defined environmental stimuli.

The secretion of T3SS effectors investigated within this thesis is an important component of bacterial colonisation, invasion, and intracellular survival and is the product of a dynamic network of regulatory factors controlling transcription of DNA to protein translation. In this chapter, RT-qPCR was employed to unravel differences at the transcriptional level between ST4/74 and SCSA50, dissecting differences in the expression of major T3SS regulators and effectors.

Between ST4/74 and SCSA50 there were no significant difference in the transcriptional expression of effectors *sipC, pipB, pipB2,* and *sifA*. The lack of significant difference between strains for *sipC* was surprising – both proteomics and western blot validation demonstrated that the protein SipC is expressed at a higher level in ST4/74. There is evidence in the literature for post-transcriptional regulation of *sipC* by transcription factor DksA (Rice *et*

al., 2015) and RNA-binding protein CsrA (Holmqvist *et al.*, 2016), and the lack of significant RT-qPCR data presented here supports these studies.

Moreover, whilst there have been no studies specifically identifying post-transcriptional regulation of T3SS-2 effectors, they are predicted to be also subject to similar post-transcriptional regulation (Holmqvist *et al.*, 2016).

These results also suggest that validation of proteomics data by RT-qPCR is an inadequate replacement for western blotting. This conclusion strengthens the distinction between this investigation and the comparative proteome study by Huang *et al.,* which demonstrated by whole-cell proteomics that virulent *S.* Choleraesuis expressed a higher level of T3SS effectors than a lab-adapted *S.* Typhimurium strain which was validated only by RT-qPCR (Huang *et al.*, 2016).

In addition to hypothesising that the genes encoding effector proteins could be post-transcriptionally regulated, the transcription of T3SS chaperone proteins was investigated to probe whether T3SS chaperone expression was linked to higher secretion of effectors since chaperones protect effectors from protein degradation in the cytoplasm (Büttner, 2012). Unfortunately no trend was discovered in this assay and there was no significant difference between ST4/74 and SCSA50 in T3SS-1 or -2 chaperones.

Transcription of the central regulators of T3SS-1 and -2 was not significantly different between ST4/74 and SCSA50. For the *hil* locus, there was a biologically relevant trend toward higher expression of T3SS-1 activators *hilA, hilC* and *hilD* and lower expression of negative regulator *hilE* in ST4/74 but no conclusive data which could correlate with reduced secretion of T3SS-1 effectors by SCSA50.

The biggest confounding factor in the analysis of the RT-qPCR data was the variation between biological replicates which indicated that more replicates would add power to the experiments. Furthermore the variation between biological replicates for several probed genes could reflect the coordinated

bistable expression of T3SS-1 by bacteria growing in culture (Hautefort *et al.*, 2003; Sturm *et al.*, 2011). And it could be argued that vital phenotypes expressed by a proportion of bacteria growing in a culture are lost by averaging gene expression across a large population (Sturm *et al.*, 2011).

Despite biologically relevant trends, no transcriptional mechanism was identified that differentiated ST4/74 and SCSA50. The data presented here suggests that a post-transcriptional mechanism may control the fate of effector and regulator gene transcripts. RNA-binding proteins (RBPs) CsrA, Hfq, and ProQ have been implicated in the global post-transcriptional control of the *Salmonella* network of virulence-associated genes (Lawhon *et al.*, 2003; Sittka *et al.*, 2008; Ansong *et al.*, 2009; Holmqvist *et al.*, 2016; Westermann *et al.*, 2019). CsrA targets and binds mRNA to prevent ribosome access, translation, and RNA stability of its target (Dubey *et al.*, 2005; Mercante *et al.*, 2009; Volk *et al.*, 2019) and data suggests that regulation of several virulence gene mRNA transcripts are dependent on the intracellular concentration of CsrA itself (Altier *et al.*, 2000; Lawhon *et al.*, 2003). Intracellular concentration of regulatory RNA-binding proteins could be a factor differentiating ST4/74 and SCSA50.

Salmonella virulence is tightly controlled by a fine-tuned network of genes that are subject to multiple layers of cascading and synchronous control in response to environmental stimuli. Whilst this investigation has not uncovered a transcriptional mechanism differentiating ST4/74 and SCSA50, it suggests that future experiments studying post-transcriptional regulation could be insightful.

Chapter 6 Invasion and intracellular survival of ST4/74 and SCSA50 within porcine epithelial cells

6.1 Introduction

Host-generalist and host-restricted or -adapted serovars are distinguished clinically by disease presentation (Table 3.5). Whilst host-generalist serovars like *S.* Typhimurium typically cause self-limiting enteritis, host-adapted *S.* Choleraesuis and host-restricted *S.* Typhi disseminate systemically in their respective natural hosts and require antibiotic treatment and frequently, hospitalisation.

S. Typhimurium and *S.* Choleraesuis pathology in swine has been characterised in ligated ileal loops and after oral challenge (Bolton *et al.*, 1999; Meyerholz and Stabel, 2003; Paulin *et al.*, 2007; Uthe *et al.*, 2007). Three studies (Bolton *et al.*, 1999; Watson *et al.*, 2000; Paulin *et al.*, 2007) challenged pigs with *S.* Typhimurium ST4/74 and *S.* Choleraesuis SCSA50, the same strains used in this thesis. *In vivo* SCSA50 invaded the porcine ileum less but was isolated in higher numbers in the extraintestinal mesenteric lymph nodes than ST4/74 (Paulin *et al.*, 2007). In the ileum SCSA50 also induced less fluid secretion, neutrophil influx and less secretion of pro-inflammatory cytokine IL-8 than ST4/74 which the authors correlated with the faster intestinal replication of ST4/74 (Paulin *et al.*, 2007).

Faster intestinal replication of ST4/74 was a promising indicator of a potential mechanism as to why SCSA50 infection results in systemic disease. As the inflamed gut is rich in nutritional resources (Stecher *et al.*, 2007; Winter *et al.*, 2010) SCSA50 may not have a competitive advantage against the commensal microbiota in the gut, especially with growing evidence that specific metabolic gene pathways are critical for intestinal proliferation (Thiennimitr *et al.*, 2011; Faber *et al.*, 2017). *S.* Choleraesuis and other related host-restricted or adapted serovars such as *S.* Typhi, *S.* Dublin and *S.* Gallinarum share a common pattern of gene loss by the formation of

pseudogenes or deletions which has impaired these important metabolic pathways to use inflammation-derived metabolites as nutrients (Nuccio and Bäumler, 2014).

Regardless of serovar, the most pivotal component of *Salmonella* pathogenesis is the invasion and colonisation of the intestinal epithelium after ingestion. The bacteria encode an abundance of adhesins which bind to epithelial receptors – the flagellum binds host cell lipids and actin (Wolfson *et al.*, 2020), Type I fimbriae bind surface mannose-rich glycoproteins (Jones *et al.*, 1995) and the giant secreted adhesin SiiE which binds MUC1 (Li *et al.*, 2019). These receptors are expressed both *in vivo* and *in vitro* by epithelial cell lines (Mariani *et al.*, 2009).

Adherence is an important step before invasion which is driven by secreted T3SS-1 effector proteins. T3SS-1 effectors direct the formation of membrane ruffles on the apical surface of cells by cytoskeletal rearrangements, atypical Rho GTPase signalling, the destabilisation of tight junctions and the activation of inflammatory pathways (reviewed in the Introduction to this thesis). Once internalised, bacteria reside within the membrane-bound *Salmonella* containing vacuole (SCV) formed during macropinocytosis. As described in the Introduction to this thesis, *Salmonella* controls the maturation of the SCV to ensure bacterial survival and proliferation.

The SCV is a modified phagosome ideal for bacterial replication and protection from anti-microbial peptides, lethal acidification (Fredlund *et al.*, 2017) and inflammasome activation (Ruiz *et al.*, 2017). Since intracellular survival is essential for systemic spread at least in murine models of disease (Fields *et al.*, 1986), regulation of the SCV may be of great significance for host-restricted or host-adapted serovars. For instance, the cellular mechanism which restricts *S.* Typhi to the human host has begun to be uncovered using murine and human cell lines.

T3SS effector proteins GtgE, SipA, SopB and SopD2 drive the biogenesis of the SCV by controlling what markers are recruited to the vacuolar membrane (Brawn *et al.*, 2007; Bakowski *et al.*, 2010; Spanò *et al.*, 2011; Spano *et al.*, 2016). *S.* Typhi cannot prevent the recruitment of Rab GTPase markers Rab29, Rab32 and Rab38 because the bacteria does not encode a functional protease GtgE or GAP SopD2 which cleave or induce disassociation of the Rabs from the SCV (Spanò *et al.*, 2011; Spano *et al.*, 2016). In murine epithelial cells and macrophages, Rab32 then interacts with BLOC-3 which leads to phagosome maturation and bacterial cell death (Baldassarre *et al.*, 2021). The reason why *S.* Typhi conversely survives Rab32/BLOC-3 induced phagosome maturation in human cells, however, is not understood.

Whilst the pathology and immune response against *S.* Choleraesuis has been studied *in vivo,* there have been few studies probing intracellular survival and proliferation in immortalised cell lines which allow delicate cellular-pathogen interactions to be unravelled, such as regulation of Rab GTPases.

A plethora of bacterial virulence factors determine intracellular survival in different cell types including the two T3SSs, ion transporters and superoxide dismutases.

Invasion results in the activation of the NF-κB pathway which results in the recruitment of the innate immune system to infected sites *in vivo* (Collado-Romero *et al.*, 2010). *In vitro*, epithelial cells and macrophages still secrete chemoattractant signals and undergo inflammasome activation and apoptosis as a defence strategy (Chen *et al.*, 1996; Hobbie *et al.*, 1997; Gewirtz *et al.*, 2001; Yu *et al.*, 2003; Rydström and Wick, 2007; Bruno *et al.*, 2009). To combat cell death and the destruction of their intracellular niche, *Salmonella* secretes a family of T3SS effectors (PipA, GtgA and GogA) to inhibit the NF-κB pathway and prolong bacterial survival (Sun *et al.*, 2016).

Both epithelial cells and phagocytes use reactive oxygen species to control bacteria within vacuoles after invasion (Mastroeni *et al.*, 2000) and limitation of essential nutrients. In accordance, *Salmonella* encodes superoxide dismutase *sodCI* which localises to the bacterial periplasm to degrade superoxides (Korshunov and Imlay, 2002). Iron, magnesium, potassium and zinc are limited nutrients within SCVs and are acquired by *Salmonella* siderophores enterobactin and salmochelin (iron) (Saha *et al.*, 2019), MgtABC proteins (Magnesium) (Blanc-Potard and Groisman, 1997; Günzel *et al.*, 2006), Trk system (potassium) (Parra-Lopez *et al.*, 1994) and ZnuABC uptake system (zinc) (Ammendola *et al.*, 2007) to scavenge ions and promote survival within the SCV.

Typically, pathogens are considered either vacuolar or cytosolic but *S. enterica* can occupy both intracellular niches (Knodler *et al.*, 2010).

Interestingly, different virulence factors and different nutrient acquisition systems are required for survival in either environment. *Salmonella* can lyse the SCV within 15 minutes after internalisation to form a cytosolic population as large as 20-30% of the total intracellular bacteria (Knodler *et al.*, 2014). Despite down-regulation of T3SS-1 after internalisation by Lon protease (Boddicker and Jones, 2004), T3SS-1 secreted effectors SipA and SopB still influence lysis of the vacuole membrane (Klein *et al.*, 2017; Chong *et al.*, 2019).

In the cytosol iron is still limited but zinc and magnesium are available (Powers *et al.*, 2021). Because of its richness in nutrients and a neutral pH, the cytosol is significantly more permissive for replication, although the bacteria are exposed to the pattern recognition receptors Nod-like and Toll-like receptors (NLRs and TLRs) which result in cascades of immune signalling (Gewirtz *et al.*, 2001; Hausmann *et al.*, 2020). *In vitro* cytosolic replication is dependent on the cultured cell type and does not occur in fibroblasts or macrophages (Beuzón *et al.*, 2002).

Cytosolic *Salmonella* express two potent antigens that vacuolar *Salmonella* do not – T3SS-1 and flagellin (Cirillo *et al.*, 1998; Hautefort *et al.*, 2008; Antonio Ibarra *et al.*, 2010; Knodler *et al.*, 2010). Autophagy is therefore a major cellular response against cytosolic bacteria since intracellular NLRs recognise *Salmonella* antigens and activate inflammasomes and caspases (Hoffmann *et al.*, 2010; Rauch *et al.*, 2017). Cells containing hyper-replicating *Salmonella* populations therefore eventually detach from monolayers and release bacteria into the extracellular environment (Knodler *et al.*, 2010; Malik-Kale *et al.*, 2012; Yu *et al.*, 2014) which, in an *in vivo* infection, could increase intestinal colonisation (Knodler, 2015).

Accordingly, whilst hyper-replicating *S. enterica* has never been profiled *in vivo*, researchers theorise that it may still be a relevant strategy for proliferation (Knodler, 2015) and of great interest to the study of ST4/74 and SCSA50 replication.

Both ST4/74 and SCSA50 showcased equivalent swimming motility in agar, as demonstrated in the first results chapter in this thesis, and the secretion of SiiE was also determined to be similar. Quantitative proteomics and western blotting, however, identified differences in other secreted proteins. T3SS-1 effectors were more abundant in the ST4/74 secretome while there were more T3SS-2 effectors secreted by SCSA50 under T3SS-1 inducing conditions. Whether these differences affect invasion, intracellular proliferation or escape to the cytosol must be investigated.

Furthermore, whilst several studies have compared the invasiveness and the activation of cytokines by *S.* Typhimurium and *S.* Choleraesuis strains (Bolton *et al.*, 2000; Skjolaas *et al.*, 2007; Veldhuizen *et al.*, 2009), no *in vitro* study has explored the intracellular lifestyles of these serovars within relevant porcine epithelial cell types.

6.2 Chapter objectives

1. To quantify the invasion and intracellular survival of ST4/74, SCSA50 and a ST4/74 T3SS-1 mutant within porcine intestinal and kidney epithelial cells.
2. To measure the replication of intracellular bacteria using plasmid partitioning.
3. To profile the intracellular cytosolic and vacuolar populations of bacteria.

6.3 Results and discussion

6.3.1 Invasion and intracellular survival of ST4/74 and SCSA50 within IPEC-J2 and PK-15 cell lines

The jejunal (IPEC-J2) and kidney (PK-15) immortal cell lines were used to represent cells derived from different porcine organs encountered by *Salmonella* bacteria during disease (Melzer *et al.*, 1965; Boyen *et al.*, 2006). IPEC-J2 cells are a spontaneously immortalised cell line harvested from the jejunum of a neonatal piglet in 1989 (Schierack *et al.*, 2006) whilst PK-15 cells were derived from the kidney of an adult pig and have been used in research since the 1960s (Harris, 1960).

Whilst cell lines cannot recapitulate *in vivo* host-pathogen interactions they can be used to explore simple interactions like cellular invasion and survival of a bacteria uncomplicated by a multicellular host. Expression of genes encoding key pathogen receptors and immune signalling by IPEC-J2 cells was assessed by qPCR and confirmed the expression of epithelial genes such as the receptor for SiiE (MUC1) and genes related to the immune response (Mariani *et al.*, 2009). The study concluded that the IPEC-J2 cell line retained, at least at the transcriptional level, its epithelial nature and was therefore a valuable model for cellular-pathogen interactions.

Invasion of IPEC-J2 cells by *S.* Typhimurium and *S.* Choleraesuis strains has been previously investigated (Schierack *et al.*, 2006; Veldhuizen *et al.*, 2009) but this was the first comparative serovar study performed using PK-15 cells.

Both IPEC-J2 studies used different strains, a different MOI and different time points but estimated that *S.* Choleraesuis invaded relatively less than *S.* Typhimurium.

The invasion of ST4/74, SCSA50 and ST4/74 Δ*prgH* was first tested after overnight culture at 25 ˚C*.* This culture method differed from the T3SS-1 inducing conditions used previously and was used prospectively since 25 ˚C culture would be required to measure the net replication of the strains (described in Figure 6.4).

To determine whether ST4/74 and SCSA50 secreted different levels of T3SS-1 effector SipC after overnight culture at 25 ˚C, cell-associated and secreted proteins were harvested from the cultures before precipitation and preparation for SDS-PAGE as previously described. Western blotting was then performed using antibodies raised against SipC and loading control DnaK (Figure 6.1A).

Thereafter gentamicin protection assays were performed in IPEC-J2 and PK-15 cells. A multiplicity of infection (MOI) of ~ 20 bacteria per host cell was used to infect cells using a 30-minute gentamicin protection assay. Bacteria were brought into contact with the confluent monolayer by centrifugation, allowed to invade for 30 minutes, before the cells were incubated with fresh media containing an inhibitory concentration of the cell-impermeable antibiotic gentamicin. After a further 30 minutes incubation, the media was removed, the cells lysed and plated on agar for enumeration of viable intracellular bacteria (expressed as a percentage of the inoculum, Figure 6.1B).

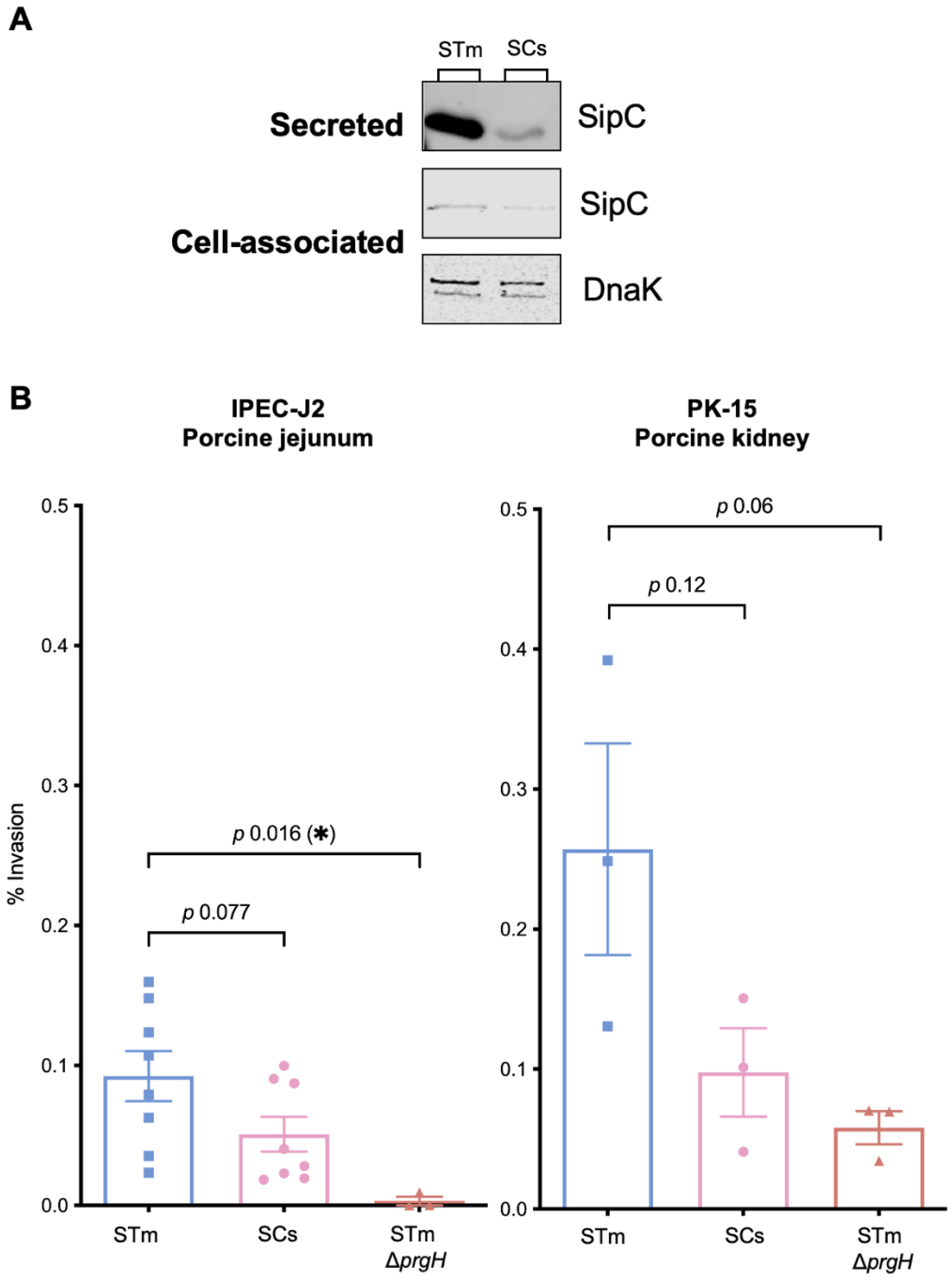


Figure 6.1 | Invasion of porcine epithelial cell lines IPEC-J2 and PK-15 by ST4/74, SCSA50 and ST4/74 Δ*prgH*

(A) The production (cell-associated) and secretion of T3SS-1 effector translocon SipC was probed by western blot after overnight culture at 25 ºC. DNA chaperone protein DnaK was used as a cell-associated loading control.

(B) 1 hour invasion with ST4/74 wild-type, SCSA50 wild-type, and ST4/74 Δ*prgH* after overnight culture at 25 ºC. Invasion % was calculated as % of initial inoculum (quantified by

retrospective plating of the viable intracellular bacteria). A student's t test was performed to calculate significance.

The inoculum, cultured overnight at 25 °C was probed for the production and secretion of SipC (Figure 6.1A). In accordance with bacteria cultured under T3SS-1 inducing conditions (Figure 4.1), ST4/74 produced and secreted more invasion protein SipC than SCSA50 (Figure 6.1A).

Surprisingly however, the invasiveness of ST4/74 and SCSA50 was not statistically significantly different when in contact with either porcine cell lines derived from different organs – the jejunum (IPEC-J2) and kidney (PK-15) when a student's t test was applied to the data (Figure 6.1B). It is appropriate, however, to note that SCSA50 did invade less than ST4/74 in both IPEC-J2 and PK-15 cells and the difference in invasiveness was approaching statistical significance in IPEC-J2 cells.

Both serovars require the activity of T3SS-1 for *in vivo* colonisation (Lichtensteiger and Vimr, 2003; Chaudhuri *et al.*, 2013; Vohra *et al.*, 2019) but have additional invasion strategies via Rck, PagN and additional unknown mechanisms (Lambert and Smith, 2008; Roche *et al.*, 2018; Mambu *et al.*, 2020). It is therefore not unreasonable to expect that invading *Salmonella* use multiple methods to enter host cells.

Moreover, to truly unpick the effect of T3SS-1 expression on SCSA50 invasion, further studies should include a T3SS-1 SCSA50 mutant and microscopy to identify potential differences in invasion strategies.

The invasiveness of the T3SS-1 null mutant, ST4/74 Δ*prgH*, was significantly attenuated compared to the wild-type strain in IPEC-J2 cells but was not in PK-15 cells. Although statistically the T3SS-1 mutant invasiveness was approaching significance in PK-15 cells, the data suggests that there may be a difference in the requirement of T3SS-dependent and -independent invasion strategies for bacteria encountering different cell types. Furthermore, in murine and bovine models of salmonellosis *Salmonella*

expresses different T3SS-1 effectors in different tissues (Gong *et al.*, 2010; Vohra *et al.*, 2019) and it is therefore notable that ST4/74 Δ*prgH* was more invasive in one cell line than another.

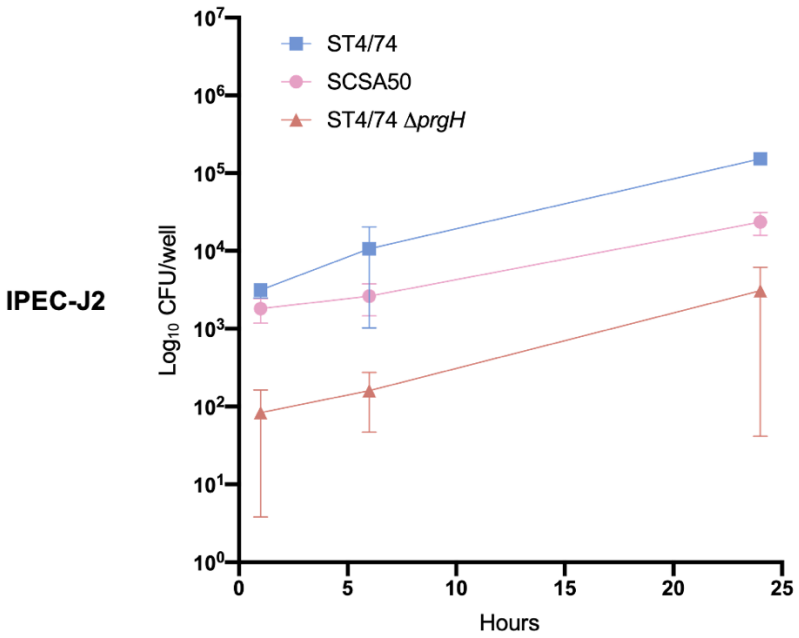
Additionally, there is evidence that T3SS-1 mutants invade cells *in vitro* (Roche *et al.*, 2018) and still colonise the gut *in vivo*, albeit with attenuation (Chaudhuri *et al.*, 2009, 2013; Vohra *et al.*, 2019). Since gentamicin protection was performed after 30 minutes, these results represent *Salmonella* invasion, and little extracellular or intracellular replication.

Several studies have compared the invasion of *S.* Typhimurium and *S.* Choleraesuis in epithelial cell lines, primary cells, and *in vivo* (Bolton *et al.*, 1999; Watson *et al.*, 2000; Schierack *et al.*, 2006; Paulin *et al.*, 2007). In IPEC-J2 invasion assays, *S.* Choleraesuis was similarly less invasive than *S.* Typhimurium (Schierack *et al.*, 2006; Veldhuizen *et al.*, 2009). In porcine ligated ileal loop experiments, the relative invasiveness of serovars did not correlate with severity of disease – SCSA50, for example, did not invade the porcine terminal ileum at a higher rate than *S.* Typhimurium which led the authors to conclude that invasion did not differentiate serovar *in vivo* virulence (Bolton *et al.*, 1999; Paulin *et al.*, 2007).

Evidence from oral and loop challenge experimentation with ST4/74 and SCSA50 (Paulin *et al.*, 2007) led to the postulation that intracellular survival was more important than invasion.

The intracellular survival of ST4/74 and SCSA50 within IPEC-J2 and PK-15 cells was next quantified after 6 hours and 24 hours post invasion (Figure 3). The CFU/well data was then plotted and the area under curve (AUC) calculated. The AUC value for each replicate ($n \geq 3$) was then used to perform an analysis of variance (ANOVA) to uncover the effect of strain on intracellular survival. A subsequent student's t test was then used to compare between ST4/74 and SCSA50 and ST4/74 and the T3SS-1 mutant ST4/74 Δ*prgH* (Figure 6.2).

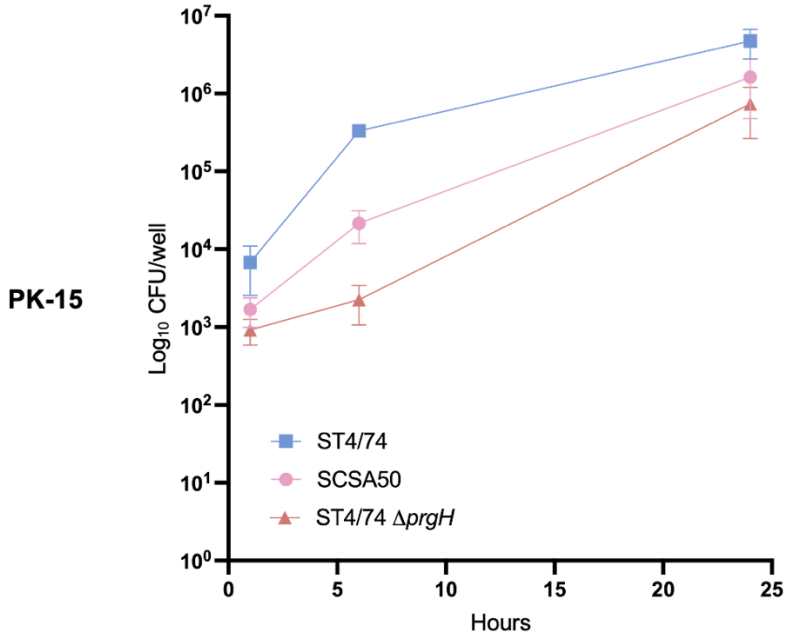
A



B

ANOVA				
Factor	**R squared**	***p* value**	***p* value summary**	**Significant?**
Strain	0.9635	<0.0001	****	Yes
Student's t test (unpaired, two-tailed)				
Comparison		***p* value**	***p* value summary**	**Significant?**
ST4/74	SCSA50	<0.0001	****	Yes
ST4/74	ST4/74 Δ*prgH*	<0.0001	****	Yes

C



D

ANOVA				
Factor	**R squared**	***p* value**	***p* value summary**	**Significant?**
Strain	0.6263	0.0522	ns	No
Student's t test (unpaired, two-tailed)				
Comparison		***p* value**	***p* value summary**	**Significant?**
ST4/74	SCSA50	0.0778	ns	No
ST4/74	ST4/74 Δ*prgH*	0.0938	ns	No

Figure 6.2 | Intracellular survival of ST4/74 and SCSA50 in porcine epithelial cell lines IPEC-J2 and PK-15.

Survival of ST4/74 wild-type, SCSA50 wild-type, and ST4/74 Δ*prgH* after 1 hour, 6 hours, and 24 hours within IPEC-J2 (A) and PK-15 (C) cells. The results are a summary of equal or more than 3 biological replicates. For each cell type the area under the curve was calculated on every replicate and used in a one-way ANOVA ascertain the impact on the strain on intracellular survival. Subsequent student's t tests were then performed. Error bars represent mean + SEM.

(A/B) Intracellular survival within IPEC-J2 cells.

(C/D) Intracellular survival within PK-15 cells.

The intracellular survival of ST4/74 and SCSA50 within IPEC-J2 and PK-15 cells over 24 hours was quantified by colony counts on LB agar. The pattern of intracellular survival of both strains differed in the different cell lines (Figure 6.2A/B) but both strains, and the T3SS-1 mutant ST4/74 Δ*prgH,* multiplied intracellularly unlike within phagocytic cells (Watson *et al.*, 2000).

In IPEC-J2 cells there was a statistically significant difference between the pattern of intracellular survival between all three strains; ST4/74 and SCSA50 and between ST4/74 and ST4/74 Δ*prgH* (Figure 6.2A/B). ST4/74 was isolated in higher numbers at all time points which suggested that the strain survived and proliferated better than SCSA50 and the Δ*prgH* mutant.

In PK-15 cells, the strains were similarly invasive but the pattern of intracellular survival differed. Intracellular survival did not differ between strains when the AUC data was interrogated by ANOVA and Student's t test (Figure 6.2C/D). This was a surprising and exciting finding – the data suggested that there were cell-type differences which influenced the intracellular survival of all strains. The data also suggested that T3SS-1 is dispensable for intracellular growth within PK-15 cells.

Murine cell lines have historically been useful for identifying differences in bacterial pathogenesis and have demonstrated that *S.* Typhimurium has a unique proteome inside different cell types encountered during an infection, the intestinal epithelium, macrophage and liver epithelium (Burns-Keliher *et al.*, 1998). Figure 6.2 supported this data and illustrated differences in patterns of *S. enterica* intracellular growth between two porcine cell lines.

The effect of cytotoxicity on the integrity of the cells was then tested since bacterial virulence factors involved in adhesion, invasion and intracellular survival promote inflammation, the destabilisation of tight junctions between cells and cellular death pathways (Köhler *et al.*, 2007; Lin *et al.*, 2020).

The release of lactate dehydrogenase (LDH), an enzyme released when the host cell membrane is damaged during a necrotic cell death pathway (Chan

et al., 2009), was thus measured and cytotoxicity calculated as a % of the total LDH within lysed uninfected cells (Figure 6.3).

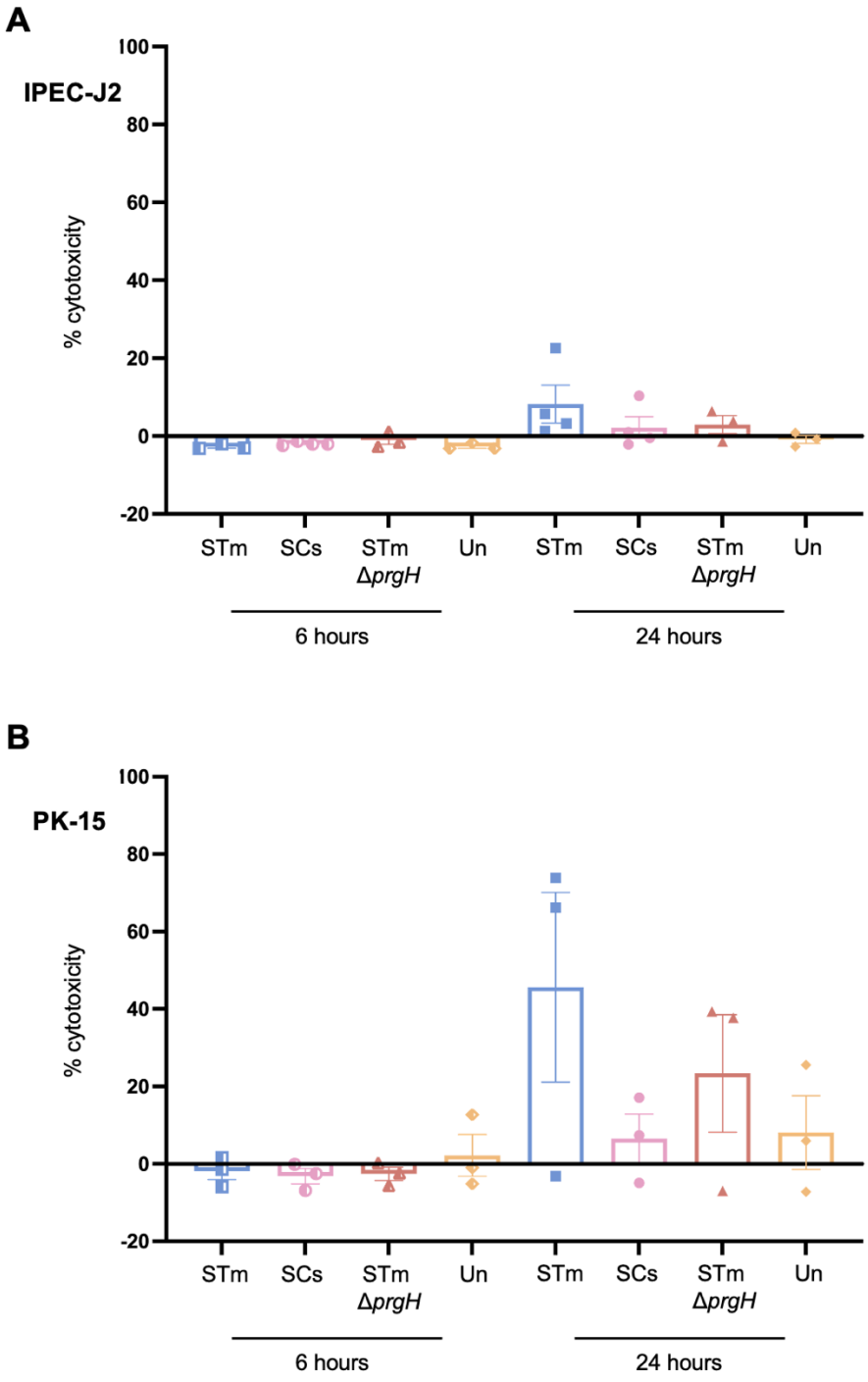


Figure 6.3 | Release of lactate dehydrogenase (LDH) by infected cells.

Percentage cytotoxicity was calculated as percentage LDH present in the media overlaying infected cells of maximum intracellular LDH. Data points represent 3 biological replicates (averaged from 3 technical replicates tested). Error bars represent mean + SEM. Un = uninfected.

A one-way ANOVA did not detect any variation between the samples infected or uninfected.

IPEC-J2 cells infected with ST4/74, SCSA50 or ST4/74 Δ*prgH* did not release a significant level of LDH than uninfected cells (Figure 6.3A). There was also little difference between serovars at either time point.

Whilst it was not statistically significant, the mean % cytotoxicity after 24 hours post infection of PK-15 cells with ST4/74 caused was ~45% cell death, which could be due to the high number of intracellular bacteria (on average ~5x10^6 ST4/74 CFU/well was quantified). Since ANOVA analysis determined that there was no difference in growth between the strains after infection of PK-15 cells (Figure 6.2C/D), cytotoxicity could be a differentiating factor if more repeats were performed.

6.3.2 Replication of ST4/74 and SCSA50 within porcine epithelial cell lines

Bacterial intracellular replication is a crucial facet of bacterial pathogenesis and for ST4/74 and SCSA50, an important differentiating factor *in vivo.* After both oral challenge and infection of calf ligated ileal loops with ST4/74 and SCSA50 transformed with low-copy number temperature sensitive pHSG422, it was discovered that ST4/74 replicated faster in the ileum than SCSA50 (Paulin *et al.*, 2007).

Plasmid pHSG422 has three antibiotic resistance genes against chloramphenicol, kanamycin and ampicillin and has a temperature sensitive origin of replication which confers plasmid replication at 25 °C (Hashimoto-Gotoh *et al.*, 1981). Therefore during infection or culture at 37 °C, the plasmid does not replicate and is titrated out of the population of bacteria by segregation.

Loss of pHSG422 during bacterial cell division can then be quantified by plating on LB agar with and without the three antibiotics to calculate the % of plasmid-bearing cells. Figure 6.4 illustrates the segregation, hereby termed plasmid partitioning, of pHSG422 at 37 °C.

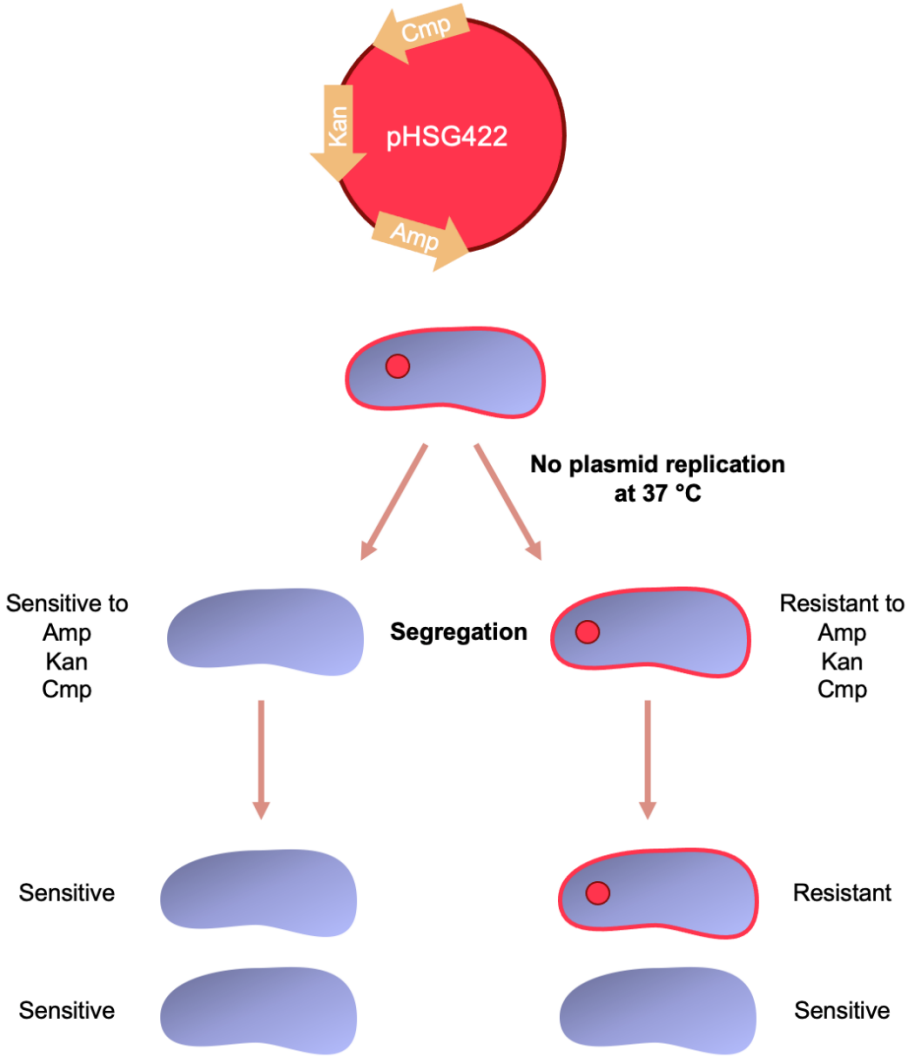


Figure 6.4 | Plasmid partitioning of temperature-sensitive pHSG422.

pHSG422 is a low-copy plasmid which does not replicate at 37 °C. As the bacteria replicate the remaining plasmid is segregated. Retention of pHSG422 can then be quantified by agar plating (with and without antibiotic pressure).

After culture, a faster replicating strain has less antibiotic-resistant pHSG422 positive recovered cells but more total colonies than a slower replicating strain which would have more pHSG422 positive colonies. Therefore differences in replication can be quantified by calculating the % plasmid bearing bacteria.

Several studies have used this method to study *Salmonella* virulence - the aforementioned *S.* Typhimurium and *S.* Choleraesuis *in vivo* comparative study (Paulin *et al.*, 2007), intracellular survival in murine dendritic cells (Jantsch *et al.*, 2003) and the effect of SPI-2 (Shea *et al.*, 1999) and *spv* genes on *in vivo* bacterial replication (Gulig and Doyle, 1993; Gulig *et al.*, 1997).

Partitioning of pHSG422 was first tested after overnight culture of strains at 25 °C in media supplemented with ampicillin, chloramphenicol and kanamycin before subculture for 4 hours in fresh LB broth at 37 °C with no antibiotics. % plasmid-bearing bacteria was calculated by enumerating bacteria on both LB agar and LB agar supplemented with antibiotics every hour (Figure 6.5).

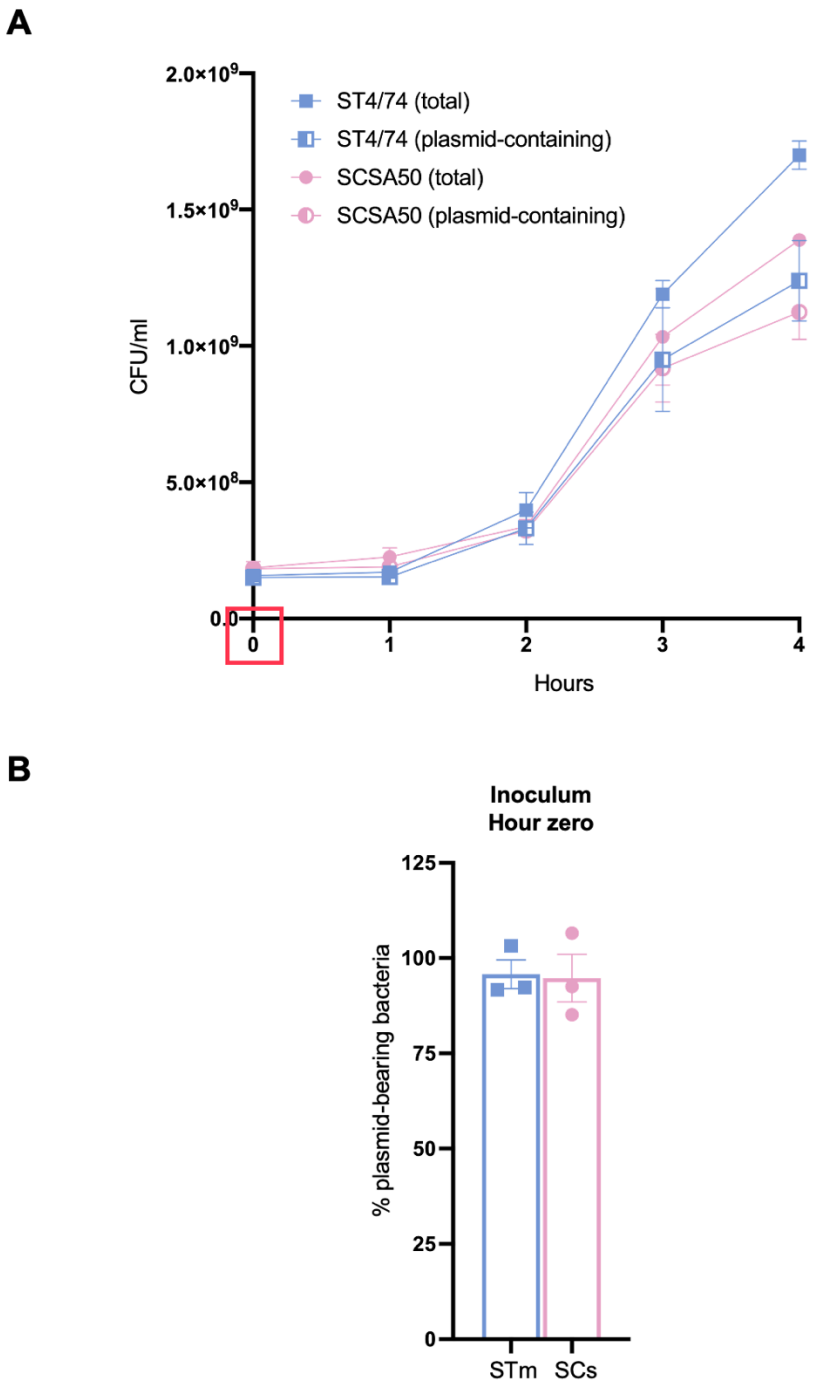


Figure 6.5 | Plasmid partitioning of pHSG422 in ST4/74 and SCSA50 after overnight culture at 25 °C.

(A) Bacteria were subcultured in LB without antibiotics at 37 °C and enumerated hourly by plating on LB agar plates with and without antibitoics to calculate % plasmid-bearing bacteria.

(B) At hour 0 (indicated by the red box in (A)), the % plasmid bearing ST4/74 and SCSA50 repersent the innoculum that would be used to infect cells.

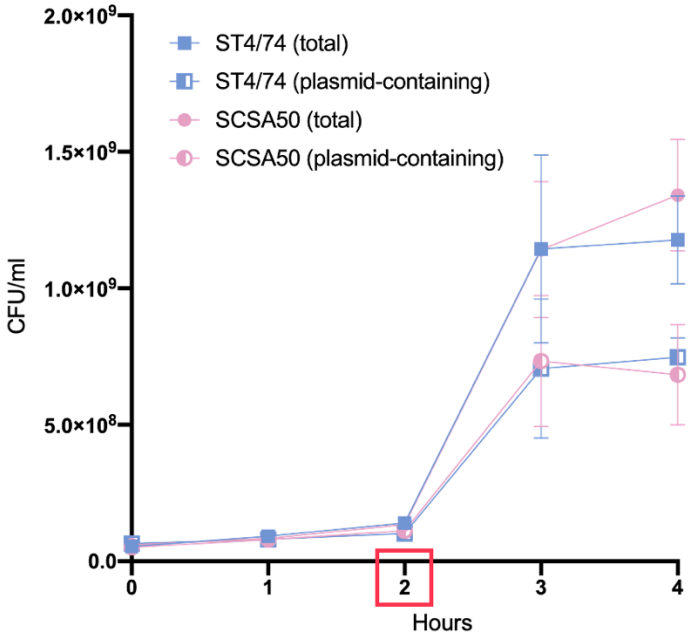
After overnight culture at 25 ˚C, approximately 100% of total ST4/74 and SCSA50 bacteria retained temperature-sensitive plasmid pHSG422 (Figure 6.5B) but began losing the plasmid after three hours subculture at 37 ˚C (Figure 6.5A). Since bacterial proliferation over time was determined by increasing CFU/ml, the data implied that the bacteria retained pHSG422 longer than predicted. This was surprising since Paulin and colleagues cultured ST4/74 and SCSA50 using the same method as above but were able to measure plasmid partitioning *in vivo* (Paulin *et al.*, 2007).

To reduce the retention of pHSG422 by bacteria after overnight culture at a temperature permissive for plasmid replication, a second method was adopted from a study which determined that *in vivo* plasmid partitioning was optimal when approximately 75% of bacteria contained pHSG422 prior to infection (Gulig and Doyle, 1993).

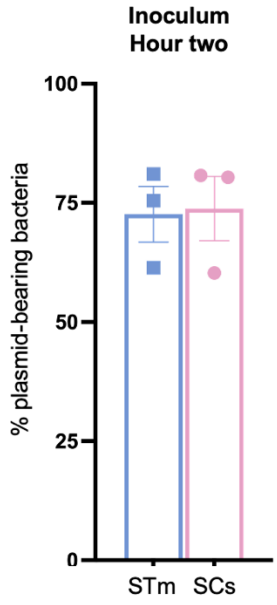
The strains were therefore cultured overnight at 30 ˚C statically with antibiotics prior to 1:10 subculture in fresh LB at 37 ˚C, shaking, without antibiotics. After two hours subculture the bacteria were used to infect IPEC-J2 and PK-15 cells at an MOI of 20, as previously described.

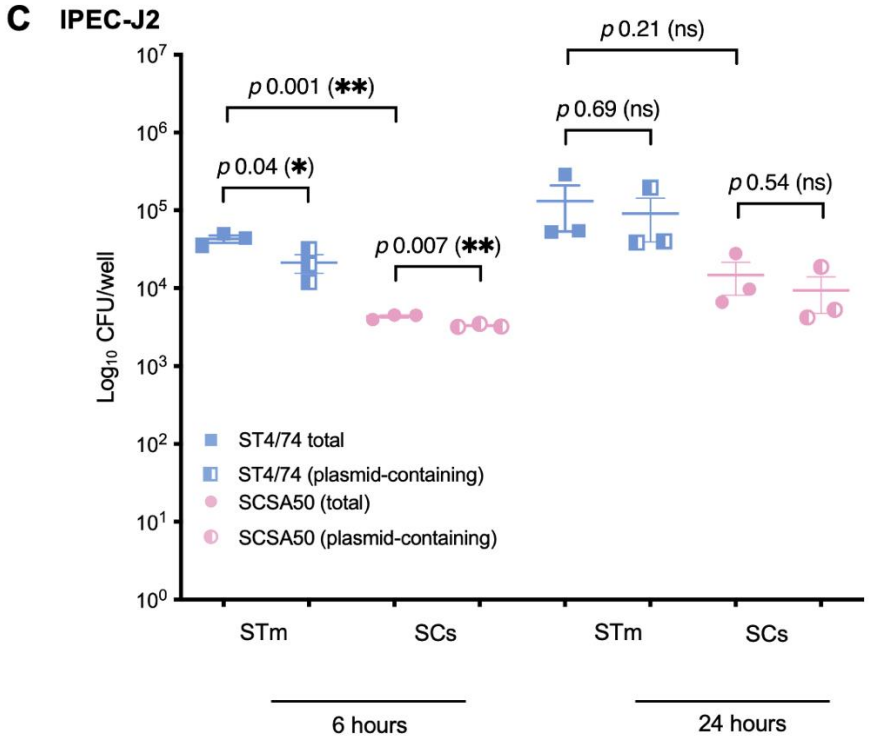
After the bacteria were brought into contact with cells and allowed to invade for 30 minutes, the media was replaced with fresh media containing gentamicin. The cells were lysed after 6 and 24 hours and intracellular bacteria were enumerated by plating on both LB agar and LB agar containing antibiotics (Figure 6.6).

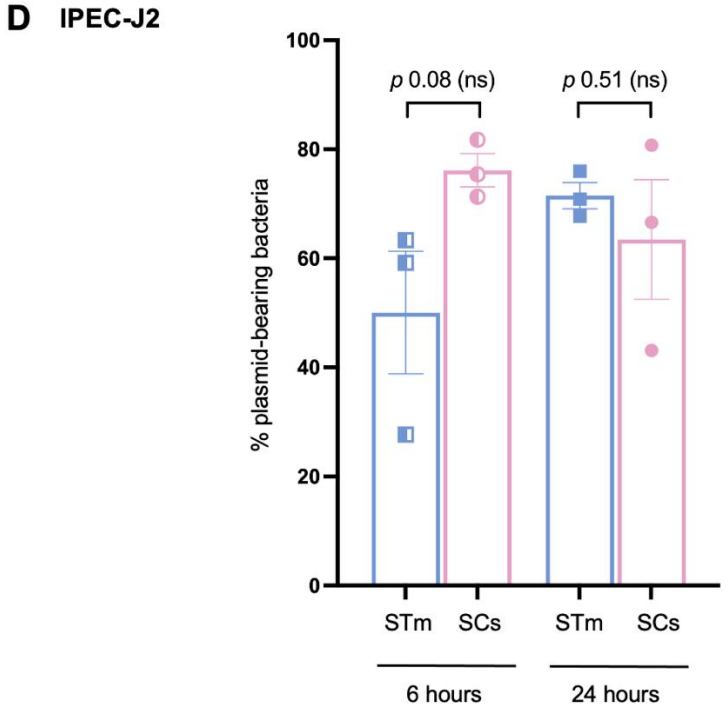
A

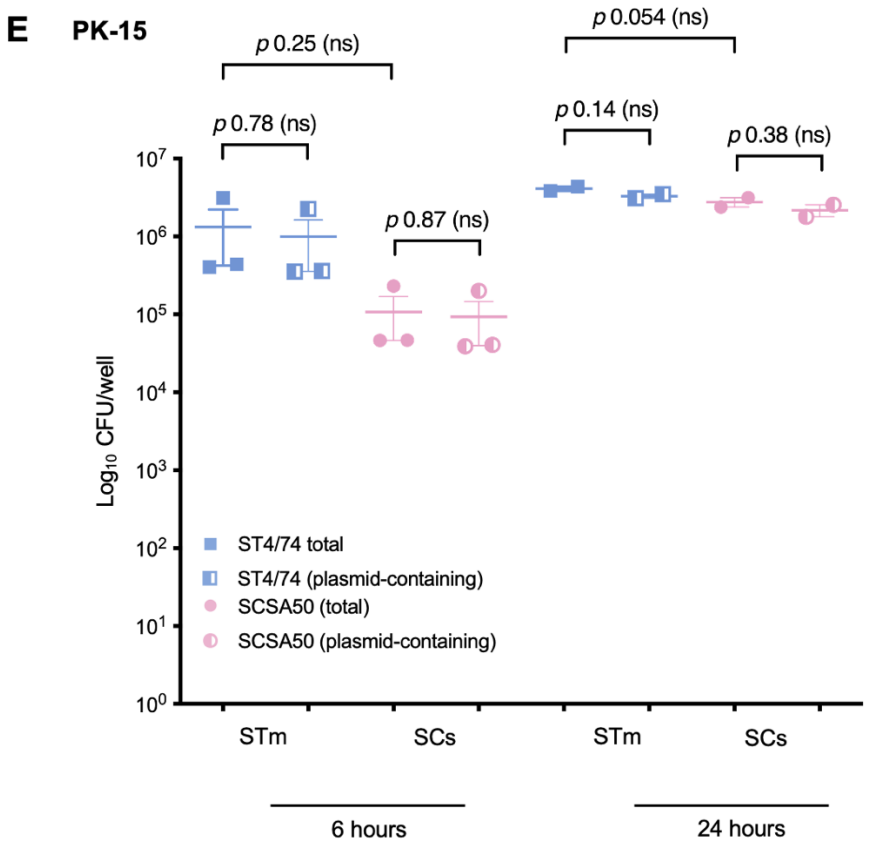

2.0×10⁹
1.5×10⁹
1.0×10⁹
5.0×10⁸
0.0
CFU/ml
ST4/74 (total)
ST4/74 (plasmid-containing)
SCSA50 (total)
SCSA50 (plasmid-containing)
0
1
2
3
4
Hours

B


Inoculum
Hour two
100
75
50
25
0
% plasmid-bearing bacteria
STm
SCs


C
IPEC-J2
p 0.21 (ns)
p 0.001 (**)
p 0.69 (ns)
p 0.04 (*)
p 0.54 (ns)
p 0.007 (**)
Log10 CFU/well
ST4/74 total
ST4/74 (plasmid-containing)
SCSA50 (total)
SCSA50 (plasmid-containing)
STm
SCs
STm
SCs
6 hours
24 hours


D
IPEC-J2
p 0.08 (ns)
p 0.51 (ns)
% plasmid-bearing bacteria
STm
SCs
STm
SCs
6 hours
24 hours


E
PK-15
p 0.25 (ns)
p 0.054 (ns)
p 0.78 (ns)
p 0.14 (ns)
p 0.38 (ns)
p 0.87 (ns)
Log10 CFU/well
ST4/74 total
ST4/74 (plasmid-containing)
SCSA50 (total)
SCSA50 (plasmid-containing)
STm
SCs
STm
SCs
6 hours
24 hours

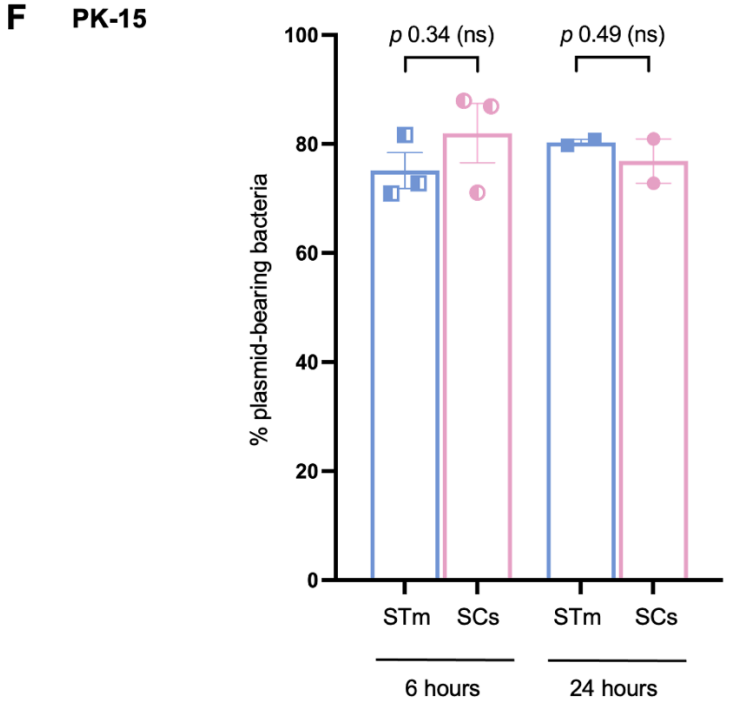

F
PK-15
p 0.34 (ns)
p 0.49 (ns)
% plasmid-bearing bacteria
STm
SCs
STm
SCs
6 hours
24 hours

Figure 6.6 | Plasmid partitioning of pHSG422 in ST4/74 and SCSA50 after overnight static culture at 30 °C.

Student's t tests were used to assess the signficance between the % retention of plasmid.

(A) Strains were cultured overnight staticly at 30 °C in the presence of antibiotics before 1:10 subculture in LB at 37 °C. Hour two is highlighted by the red box as representative of the innoculum used to infect cells.

(B/C) Partitioning of pHSG422 after invasion of IPEC-J2 cells.

(D/E) Partitioning of pHSG422 after invasion of PK-15 cells.

Overnight static culture of the strains and subsequent subculture induced measurable plasmid partitioning (Figure 6.6A). Following the method by (Gulig and Doyle, 1993), approximately 75% of both cultures retained pHSG422 at the time of infection (Figure 6.6A).

After six hours post infection of IPEC-J2 cells, SCSA50 replicated slower than ST4/74 with a significantly different number of viable bacteria isolated at this time point (Figure 6.6B). When the % plasmid bearing bacteria of the total population was calculated the data was approaching significance (Figure 6.6C). After 24 hours infection of IPEC-J2 cells, however, the strains appeared to replicate similarly.

After infection of PK-15 cells for 6 hours or 24 hours, partitioning appeared to be similar in ST4/74 and SCSA50 (Figure 6.6E). Partitioning was also not as distinguishable after PK-15 infection. On average 50% of intracellular ST4/74 contained pHSG422 within IPEC-J2 cells at 6 hours while 75% of ST4/74 within PK-15 cells contained the plasmid at the same time point. This was a fascinating finding and could be compared to data generated using the fluorescence dilution approach which demonstrated that a proportion of intracellular *Salmonella* within macrophages were viable but did not replicate (Helaine *et al.*, 2010). The data in Figure 6.6 suggest that both ST4/74 and SCSA50 may not replicate as well in PK-15 cells than IPEC-J2 cells.

The differences in net replication of ST4/74 and SCSA50 after 6 hours in IPEC-J2 cells (Figure 6.6C) can be compared to the 2007 comparative study in pigs which demonstrated by plasmid partitioning that ST4/74 replicated faster in the porcine ileal mucosa than SCSA50 (Paulin *et al.*, 2007). The study also identified potential differences in replication of the strains in different locations – the bacteria appeared to replicate slower in the colonic lymph nodes than the ileal lymph nodes (Paulin *et al.*, 2007). This data could support the hypothesis that the strains replicate differently in different cell types.

It also must additionally be commented that the *in vivo* swine ileum or kidney contains a myriad of cell types that are missing from simple experiments in an immortalised cell line which could influence replication dynamics.

Paulin and colleagues postulated that differences in net replication was related to differences in inflammation of the porcine ileal mucosa – ST4/74 replicated faster and therefore induced a stronger inflammatory response – which was further linked to increased secretion of T3SS-1 effector SipC by ST4/74 (Paulin *et al.*, 2007). The role of T3SS activity on bacterial replication is well understood for T3SS-2 (Salcedo and Holden, 2003) but T3SS-1 was additionally found to be important for intracellular replication in the 3D intestinal cell line HT-29 (Radtke *et al.*, 2010). Whilst cell lines cannot recapitulate the *in vivo* interactions between the host and bacteria, infections of a well-characterised epithelial cell line for assaying bacterial replication in the context of gene deletion mutations, for example, could be useful to inform on the necessity of animal experimentation.

A future study could include fluorescence dilution as a secondary technique to study bacterial replication. The technique utilises progressive loss of plasmid-derived GFP signal in replicating bacteria by flow cytometry (Helaine *et al.*, 2010). This technique has previously been used to show intracellular bacterial replication within macrophages (Vohra *et al.*, 2019) and could be compared as a technique to plasmid partitioning to study replication of

ST4/74 and SCSA50 in epithelial cells. Moreover, the plasmid partitioning data could be more meaningful with more replicates and potentially several more time points.

6.3.3 Cytosolic and vacuolar populations of intracellular *S. enterica*

The intracellular replicative niche of *Salmonella* bacteria is often considered the SCV which is anchored close to the host Golgi body and nucleus (Salcedo and Holden, 2003). There is, however, a population of bacteria that escape the vacuole to the host cytosol which is more permissive to faster rates of replication (Knodler *et al.*, 2010). T3SS-1 effectors SipA and SopA have a role in vacuole lysis (Klein *et al.*, 2017; Chong *et al.*, 2019) as soon as 15 minutes after internalisation (Knodler *et al.*, 2014). Several studies have used microscopy to profile intracellular epithelial cell infections 4-8 hours after *S.* Typhimurium inoculation and discovered 20-30% of the total intracellular population is cytosolic and T3SS-1 active after 8 hours (Knodler *et al.*, 2014).

As promising plasmid partitioning data suggested the faster replication of ST4/74 bacteria in IPEC-J2 cells after 6 hours (Figure 6C/D) and in the porcine ileum during *in vivo* experimentation (Paulin *et al.*, 2007), the quantification of the cytosolic population was performed to decipher differences between ST4/74, SCSA50 and the T3SS-1 mutant ST4/74 Δ*prgH.*

A chloroquine resistance assay was planned in concert with gentamicin protection. Chloroquine is a lysosomotropic drug which selectively kills vacuolar bacteria but does not access cytosolic bacteria (Steinberg, 1994; Rolain *et al.*, 2007). The experiment is described in Figure 6.7.

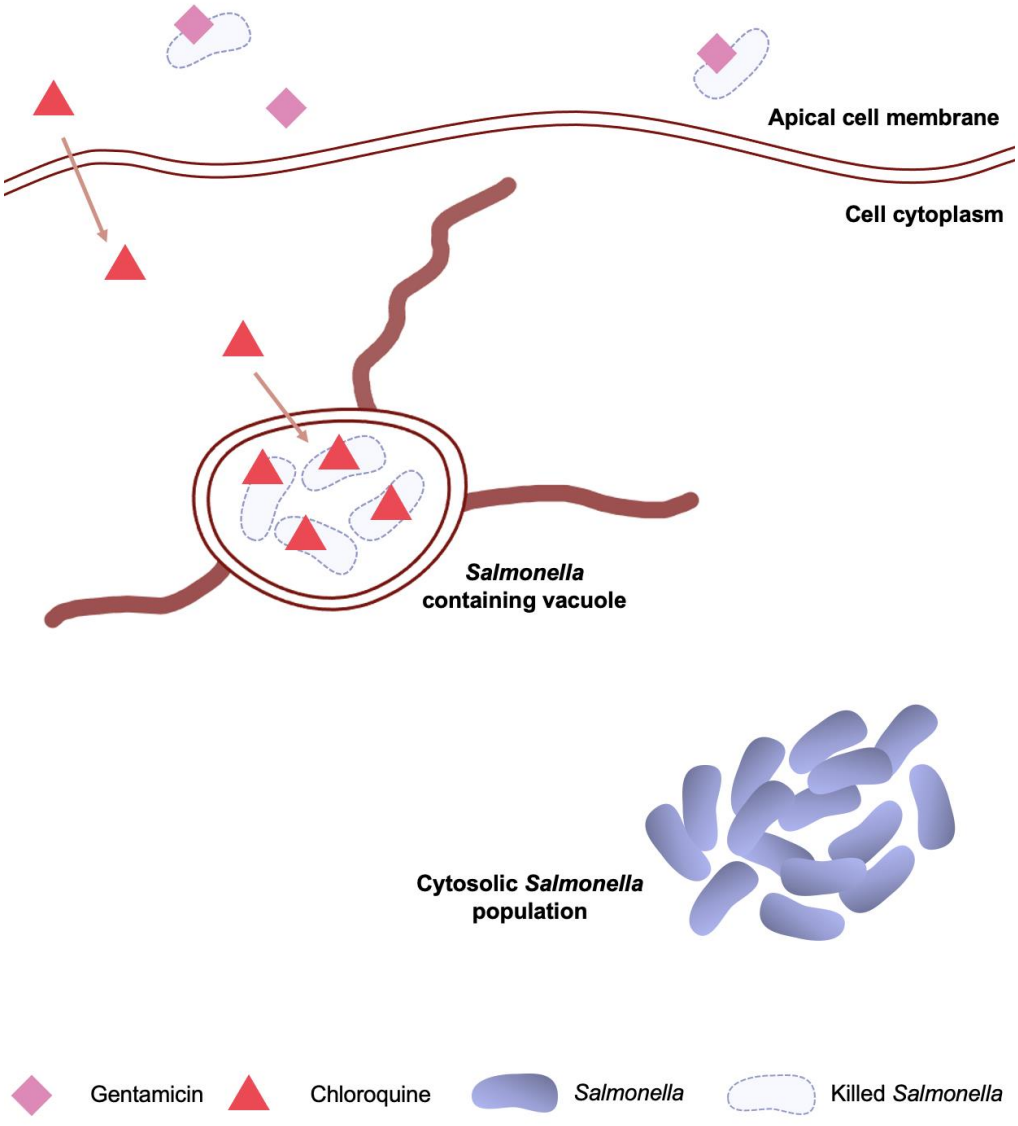


Figure 6.7 | Chloroquine resistance assay.

Chloroquine resistance is performed in concert with gentamicin protection. Chloroquine is a drug which becomes intracellular but is trafficked by the endocytic pathway to the *Salmonella* containing vacuole. Any cytosolic bacteria are not killed.

The technique was thus performed in cell lines IPEC-J2 and PK-15 to investigate the intracellular lifestyle of ST4/74 and SCSA50 and differences between the two cell types.

Bacteria were cultured overnight, as previously described at 25 ˚C. Firstly a 30-minute gentamicin protection assay was performed and after a further 6 hours (6.5 hours after initial infection), the overlaying media was replaced with fresh media containing either both gentamicin and 50 μM chloroquine or gentamicin alone for 1.5 hours. Therefore, a chloroquine resistance assay was performed to distinguish intracellular populations of cytosolic and vacuolar bacteria after eight hours infection firstly in IPEC-J2 cells (Figure 6.8).

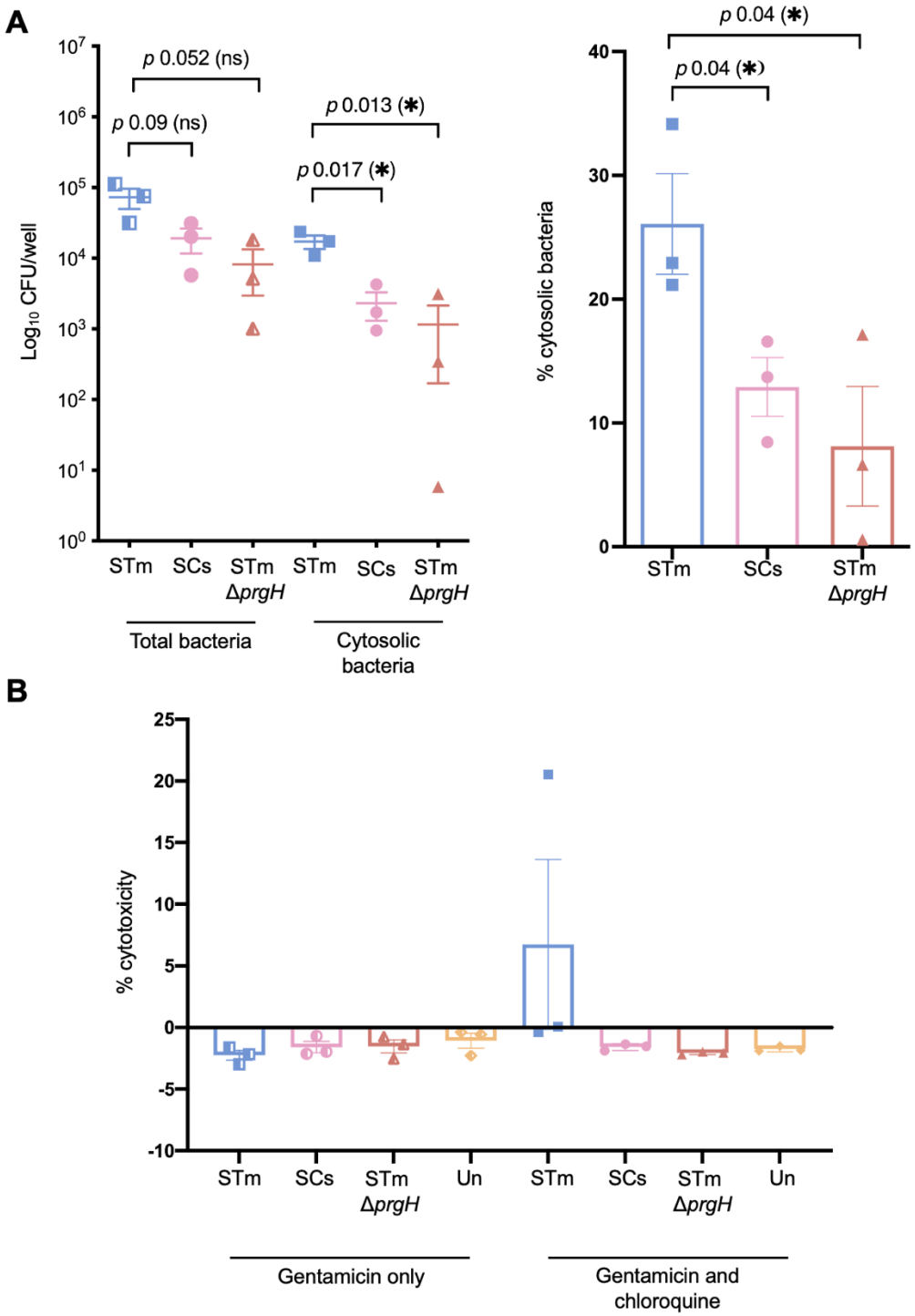


Figure 6.8 | The cytosolic populations of ST4/74 and SCSA50 in IPEC-J2 cells.

Strains were cultured overnight as previously descrbied and a gentamicin protection assay perfromed at 30 minutes post infection. At hour 6.5 either gentamicin alone (total bacteria) or gentamicin and chloroquine (cytosolic bacteria alone surivve) was added to the cells. At hour 8, the cells were lysed to enumerate viable intracellular bacteria on agar. Error bars represent mean + SEM.

(A) CFU/well calcualted from the bacteria isolated from wells. CFU/well data was then used to calcuale the % of the intracellular population which was cytosolic. A student's t test was used to calculate signficance.
(B) LDH release assay was performed on all repeats. A one-way ANOVA test determined that there was no signficant difference between the wells.

Significantly less intracellular SCSA50 were cytosolic than ST4/74 (Figure 8A). There was also significantly less intracellular and cytosolic ST4/74 Δ*prgH* bacteria. These differences were not due to cytotoxicity of drug treatment or bacterial infection (Figure 6.8B).

A larger population of ST4/74 in the cytosol could be a result of increased expression of the T3SS-1 effectors linked to vacuole escape (Klein *et al.*, 2017; Chong *et al.*, 2019) which could correlate with data previously presented in this thesis (Figure 3.7). The bacterial genes required for intracellular survival, vacuole maintenance and vacuole lysis are vastly different from genes relevant for bacterial culture in rich laboratory media, however.

The ST4/74 data supported earlier experiments which quantified the cytosolic population of intracellular *S.* Typhimurium to be between 20-30% (Knodler *et al.*, 2014; Knodler, 2015). The significant reduction in cytosolic ST4/74 Δ*prgH* relative to wild-type ST4/74 demonstrated the importance of T3SS-1 to vacuole escape, also demonstrated by other studies (Klein *et al.*, 2017; Chong *et al.*, 2019).

The smaller % of cytosolic intracellular SCSA50 was a novel finding and could provide mechanistic insight into differential replication dynamics between the strains in IPEC-J2 cells (Figure 6.6C) since the host cell cytosol is significantly more permissive to bacterial replication. As hypothesised above, the reduced secretion of proteins such as SipA and SopB by SCSA50 in culture (Figure 4.1) could be the cause for reduced vacuole escape of SCSA50. Proteomics also demonstrated that SCSA50 secreted more T3SS-

2 protein SifA, an effector which has a role in maintenance of the vacuole integrity (Beuzon *et al.*, 2000; Knodler *et al.*, 2014).

The chloroquine resistance assay was repeated in PK-15 cells (Figure 6.9) where replication dynamics between ST4/74 and SCSA50 were similar (Figure 6.6E).

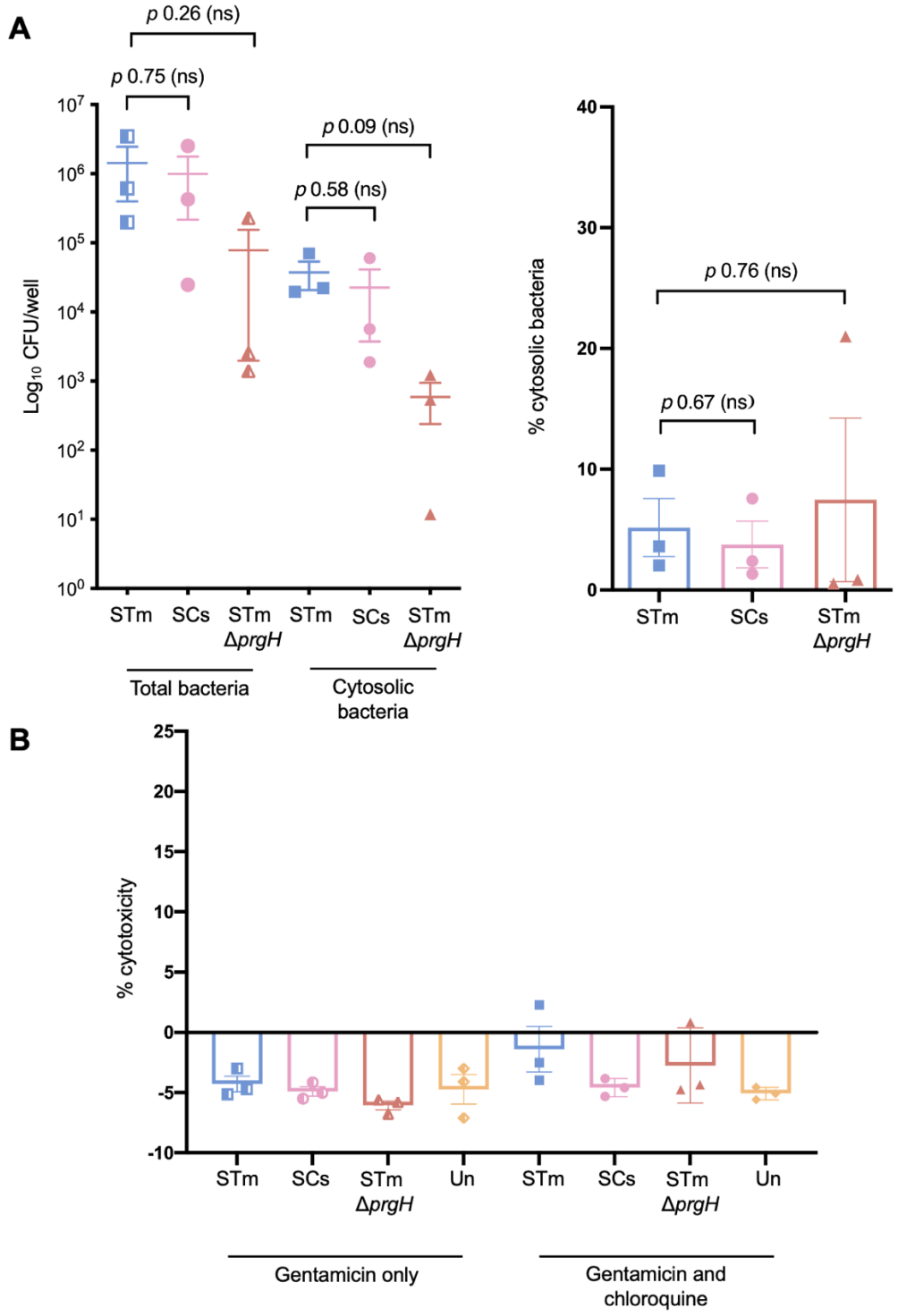


Figure 6.9 | The cytosolic populations of ST4/74 and SCSA50 in PK-15 cells.

Strains were cultured overnight as previously descrbied and a gentamicin protection assay perfromed at 30 minutes post infection. At hour 6.5 either gentamicin alone (total bacteria) or gentamicin and chloroquine (cytosolic bacteria alone surivve) was added to the cells. At hour 8, the cells were lysed to enumerate viable intracellular bacteria on agar. Error bars represent mean + SEM.

(A) CFU/well calcualted from the bacteria isolated from wells. CFU/well data was then used to calcuale the % of the intracellular population which was cytosolic. A student's t test was used to calculate signficance.

(B) LDH release assay was performed on all repeats. A one-way ANOVA test determined that there was no signficant difference between the wells.

In contrast with IPEC-J2 cells, all strains had a small cytosolic population of 5-10% of the total intracellular bacteria after infection of PK-15 cells (Figure 6.9). Evidently, there is a cell-type difference between the two cell lines.

This could be due to reduced escape from the vacuole in PK-15 cells, or it could be a result of enhanced control of the cytosolic population by the host (Chen *et al.*, 1996; Hobbie *et al.*, 1997; Gewirtz *et al.*, 2001; Yu *et al.*, 2003; Rydström and Wick, 2007; Bruno *et al.*, 2009). Despite no cytotoxicity reported, it would be of interest to study the cytosolic and vacuolar bacterial populations over time.

Overall, the chloroquine protection assays revealed that ST4/74 has a significantly higher population of cytosolic bacteria within IPEC-J2 cells than both SCSA50 and the T3SS-1 null mutant ST4/74 Δ*prgH* (Figure 6.8) but that in a different, extraintestinal cell type (PK-15), 90-95% of all three strains resided within vacuoles (Figure 6.9).

This was a fascinating result which suggests that host responses like autophagy and inflammasome activation should be screened for which could be the reason for the differential induction of inflammation *in vivo* between the strains (Paulin *et al.*, 2007).

Further work should include microscopy to identify and confirm the cytosolic localisation of bacteria and techniques such as plasmid partitioning (Figure 6.4) or fluorescence dilution (Helaine *et al.*, 2010) to measure the replication of bacteria in different cellular niches.

6.4 Final conclusions

The aim of this chapter was to ascertain differences in the invasion and intracellular lifestyles of ST4/74 and SCSA50 in contact with two porcine epithelial cell lines which represented both intestinal and extraintestinal tissue.

ST4/74 survived better than SCSA50 and T3SS-1 mutant ST4/74 Δ*prgH* in IPEC-J2 cells but not in PK-15 cells despite statistically similar invasiveness. This was an important result which illustrated a cell-type intracellular survival phenotype. Assessment of replication dynamics between the strains revealed no significant difference but a trend toward faster replication of ST4/74 in IPEC-J2 cells alone.

Replication dynamics are an important factor in the study of intracellular survival since enumerating total viable bacteria cannot discern the complex homeostasis between the host response and replicating microbes. To increase the robustness of this finding other techniques must be used to evaluate *in vitro* plasmid partitioning.

Profiling cytosolic and vacuolar intracellular bacteria at a population level also revealed differences which were cell-type specific. In intestinal-derived IPEC-J2 cells, the % of intracellular ST4/74 in the cytosol was significantly higher than SCSA50 and the *prgH* mutant. This data importantly corresponded with other chloroquine resistance studies using *S.* Typhimurium (Knodler *et al.*, 2010). It also validated the requirement of T3SS-1 activity for vacuolar escape, as determined by several studies (Klein *et al.*, 2017; Chong *et al.*, 2019).

In kidney-derived PK-15 cells however, 90-95% of intracellular bacteria resided within the SCV regardless of the strain. This was a fascinating result and was unexpected. Cell-type specific vacuole lysis and survival has been investigated by *S.* Typhimurium but only fibroblasts and macrophages

identified as cells not permissive to a replicating cytosolic population (Beuzón *et al.*, 2002; Knodler *et al.*, 2014).

The differences in intracellular survival and vacuole lysis between ST4/74 and SCSA50 specifically in intestinal epithelial cells was a novel finding and provide promising insight into SCSA50 cellular and potentially *in vivo* pathogenesis.

Future work must include studying whether cytosolic escape affects replication dynamics of ST4/74 and SCSA50 since the cytosol permits hyper-replication. Either plasmid partitioning (Figure 6.4) or fluorescence dilution (Helaine *et al.*, 2010) should be employed in further population based experiments. Whilst the cytosol permits faster replication, the bacteria are then exposed to autophagy (Birmingham *et al.*, 2006), ubiquitination (Perrin *et al.*, 2004) and inflammasome-mediated cell death (Knodler *et al.*, 2010, 2014).

The inflammasome is a key component of cytosolic control of bacteria and its activation leads to caspase-mediated cell death. The sub-Saharan invasive *S.* Typhimurium ST313 invaded epithelial cells less and induces less IL-1β and cell death in human and murine macrophages which was attributed to reduced inflammasome activation than *S.* Typhimurium strain SL1344 which is closely related to ST4/74 (Carden *et al.*, 2015). The authors commented that reduced inflammasome activation and inflammation in ST313, which like *S.* Choleraesuis escapes the intestines to become systemic, influenced systemic spread.

Screening the induction of host responses by ST4/74 and SCSA50 by staining for markers of cell death and inflammation would be an important finding since the strains result in clinically distinct immune response *in vivo*. SCSA50 induces less IL-8, less neutrophil recruitment and less fluid secretion compared to ST4/74. While cytosolic populations of bacteria have

been mentioned only anecdotally *in vivo*, differential vacuole escape could influence the *in vivo* virulence of ST4/74 and SCSA50.

The cell type differences regarding intracellular survival and vacuole escape suggest that a future *in vitro* approach should include cells from other extraintestinal sites that SCSA50 disseminates to like the liver or lung to investigate dependence on the T3SS-1 for survival is specific for cells of the intestines.

Overall, there were several novel findings related to the cellular interactions of ST4/74 and SCSA50 discovered in this chapter which lead to new questions surrounding inflammation, the environmental niche within the host and replication dynamics.

Chapter 7 General discussion and future outlook

Salmonella enterica is a zoonotic pathogen of global importance and despite its discovery 140 years ago, every new finding both leads to a greater insight into its biology but also to new questions.

The principal aim of this thesis was to investigate molecular mechanisms controlling the differential virulence of *S.* Typhimurium and *S.* Choleraesuis, two serious pathogens in swine and man which cause distinct clinical syndromes. *S.* Typhimurium infection results in self-limiting enteritis with inflammation and fluid secretion localised to the intestines. *S.* Choleraesuis conversely causes little enteric symptoms but disseminates systemically to cause bacteraemia, pneumonia and a higher mortality rate.

In this thesis, differential secretion of virulence factors by ST4/74 and SCSA50 was identified as a major finding. *S. enterica,* like many members of the Enterobacteriace, secretes virulence factors to exert control over host cells. Secreted proteins of the *Salmonella* type III secretion systems promote and perpetuate bacterial invasion and intracellular survival by manipulating host cell processes.

Staining of SDS-PAGE gels, quantitative proteomics and validating western blotting demonstrated the distinct secretion profiles of ST4/74 and SCSA50 cultured under conditions relevant for invasion. This was a novel comparative screen and identified the significantly higher production and secretion of proteins secreted by the *S. enterica* T3SS-1 which functions to promote invasion and inflammation. In contrast, SCSA50 secreted more proteins which, *in vitro* and *in vivo*, function to promote bacterial survival within the *Salmonella* containing vacuole (SCV) and systemic dissemination than ST4/74.

Validation was limited by antibody availability and growing interest in the intracellular lifestyle of ST4/74 and SCSA50 emphasises the need to extend

validation western blotting for additional proteins. SpvC, for example, was a promising protein more abundant in the SCSA50 secretome and is key to reduction in intestinal inflammation and systemic dissemination (Haneda *et al.*, 2012; Vohra *et al.*, 2019). There are also lasting questions over whether the hypothetical proteins identified are secreted and translocated by either *Salmonella* T3SS.

Whilst analysis of the genomes and predicted protein translations could not have predicted the differential secretion of T3SS effector proteins, the bioinformatic strategy identified multiple effectors absent from the genome of SCSA50. SopE and SopA, either absent or truncated in the genome of SCSA50, are critical effectors controlling the inflammation and fluid secretion characteristic of *S.* Typhimurium gastroenteritis disease.

A further functional approach could elucidate further insight into the meaningfulness of these findings. For example, T3SS-1 has the ability to form pores in host plasma membranes to facilitate invasion (Miki *et al.*, 2004) and testing the ability of both strains to lyse erythrocytes would directly measure the impact of differential secretion. Since *S.* Choleraesuis causes bacteraemia whilst disseminating during a natural infection, this would be an interesting comparative experiment to complete.

The next aim of this work was to unravel regulatory differences between the strains. RT-qPCR was used to measure the transcription of major T3SS-1 and T3SS-2 regulators after the induction of T3SS-1 but, surprisingly, there were no significant differences between the strains. Transcription of the negative regulator of T3SS-1, *hilE,* was remarkable, however. On average, SCSA50 transcribed more *hilE* than ST4/74 but the data was confounded by variation between biological replicates. More repeats could lead to the identification of a regulatory difference that could be manipulated for infection in cells or an animal model.

Post-transcriptional regulation was also hypothesised to control the differences in secretion between the strains. Lon protease, a post-translational regulator, notably represses T3SS-1 while inducing expression of T3SS-2 (Song *et al.*, 2019). Since SCSA50 secreted more T3SS-2 effector proteins than ST4/74, analysing the effect of *lon* gene deletions on virulence factor secretion could lead to a greater understanding of differences post-transcriptionally. Additional time points to assess whether degradation of RNA or translated regulators affected the RT-qPCR experiments and distinct secretion profiles of ST4/74 and SCSA50 would also be meaningful.

The last aim of this thesis was to study interactions of ST4/74 and SCSA50 with porcine epithelial cells. Two epithelial cell lines, one derived from the intestines of a neonatal piglet (IPEC-J2) and one from the kidney of an adult pig (PK-15) were used to represent two sites of interest during salmonellosis – the intestines and an extraintestinal systemic site.

Invasion was relatively similar in both cell lines, although ST4/74 was more invasive than SCSA50. Intracellular survival, however, differed. ST4/74 survived better in IPEC-J2 cells than SCSA50 or a T3SS-1 mutant but in PK-15 cells, there was no effect of strain on intracellular survival. Fascinatingly, T3SS-1 was dispensable for intracellular survival in PK-15 cells alone. This was a fascinating finding since SCSA50 is more likely to reach the extraintestinal site of the kidney during an infection.

The replication of the strains *in vivo* is an important pathological difference and reduced replication of SCSA50 was correlated with reduced intestinal inflammation and thus dissemination (Paulin *et al.*, 2007). Loss of a temperature sensitive triple-antibiotic resistant plasmid over time was used to quantify replication in IPEC-J2 and PK-15 cells. The assessment of differential replication dynamics *in vitro* was time-dependent and cell-type dependent. ST4/74 replicated relatively faster in an earlier time point than SCSA50 in IPEC-J2 cells but replication appeared to be similar in the PK-15

cell line. Further experiments using other methods such as fluorescence dilution could add to the robustness of these findings.

There was also cell-type and strain specific cytosolic escape of the strains. The % of intracellular ST4/74 residing in the replication-permissive cytosol of IPEC-J2 cells was significantly higher than SCSA50 or the T3SS-1 *prgH* mutant. In PK-15 cells, 90-95% of all strains resided in the SCV. Since cytosolic escape exposes bacteria to the host immune response and inflammatory cell death, cytosolic escape could be an important virulence strategy for ST4/74 to induce more inflammation and replication in the intestines than SCSA50.

Overall, the cellular-bacteria interactions studied suggest that ST4/74 and SCSA50 have different intracellular lifestyles in specifically intestinal epithelial cells and more work must be done to understand the relevance of T3SS on these interactions by constructing SCSA50 T3SS-1 null mutations in addition to profiling the immune response and replication of the bacteria during cytosolic escape. The intracellular survival, replication and cytosolic escape data suggests that further work could include cell types derived from a variety of organs or the use of multi-cellular organoid cultures to truly explore whether the cellular pathogenesis of the strains differs in different environmental niches.

In conclusion, this study has revealed multiple striking differences between *S.* Typhimurium strain ST4/74 and *S.* Choleraesuis strain SCSA50 including the distinct secretion profiles of T3SS effectors, intracellular survival and vacuole escape could be the mechanisms controlling the differential intestinal virulence of ST4/74 and SCSA50. However, as Theobald Smith stated in his 1900 publication,

“To admit that pathogenic forms can arise in short spaces of time is to greatly underrate the problem of parasitism and to overlook the existence of a complex relationship between host and parasite, because we cannot comprehend it.”
(Smith, 1900)

The results in this thesis emphasise that study of the host response in this study was lacking. To determine whether induction of inflammation, the hypothesised reason why serovars such as SCSA50 escape from the intestines, is controlled by the mechanisms uncovered in this study requires further work including identification of a differential regulator and the involvement of animal experimentation.

References

Abrahams, G. L. and Hensel, M. (2006) 'Manipulating cellular transport and immune responses: Dynamic interactions between intracellular *Salmonella enterica* and its host cells', *Cellular Microbiology*, 8(5), pp. 728–737. doi: 10.1111/j.1462-5822.2006.00706.x.

Abshire, K. Z. and Neidhardt, F. C. (1993) 'Analysis of proteins synthesized by *Salmonella* Typhimurium during growth within a host macrophage', *Journal of Bacteriology*, 175(12), pp. 3734–3743.

Achtman, M. *et al.* (2013) 'Population structures in the SARA and SARB reference collections of *Salmonella enterica* according to MLST, MLEE and microarray hybridization', *Infection, Genetics and Evolution*, 16. doi: 10.1016/j.meegid.2013.03.003.

Akeda, Y. and Galán, J. E. (2004) 'Genetic Analysis of the *Salmonella enterica* Type III Secretion-Associated ATPase InvC Defines Discrete Functional Domains', *Journal of Bacteriology*. doi: 10.1128/JB.186.8.2402-2412.2004.

Akoachere, J. F. T. K. *et al.* (2009) 'Phenotypic characterization of *Salmonella* Typhimurium isolates from food-animals and abattoir drains in Buea, Cameroon', *Journal of Health, Population and Nutrition*, 27(5). doi: 10.3329/jhpn.v27i5.3637.

Ali, S. A. and Steinkasserer, A. (1995) 'PCR-ligation-PCR mutagenesis: A protocol for creating gene fusions and mutations', *BioTechniques*.

Alikhan, N. F. *et al.* (2011) 'BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons', *BMC Genomics*. doi: 10.1186/1471-2164-12-402.

Allenspach, E. J. *et al.* (2008) 'Migratory and Lymphoid-Resident Dendritic Cells Cooperate to Efficiently Prime Naive CD4 T cells', *Immunity*, 29(5). doi: 10.1016/j.immuni.2008.08.013.

Althouse, C. *et al.* (2003) 'Type 1 Fimbriae of *Salmonella enterica* Serovar Typhimurium Bind to Enterocytes and Contribute to Colonization of Swine In Vivo', *Infection and Immunity*, 71(11). doi: 10.1128/IAI.71.11.6446-6452.2003.

Altier, C., Suyemoto, M. and Lawhon, S. D. (2000) 'Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by csrA', *Infection and Immunity*, 68(12), pp. 6790–6797. doi: 10.1128/IAI.68.12.6790-6797.2000.

Altschul, S. F. *et al.* (1990) 'Basic local alignment search tool', *Journal of Molecular Biology*, 215(3), pp. 403–410. doi: 10.1016/S0022-2836(05)80360-

Ammendola, S. *et al.* (2007) 'High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*', *Infection and Immunity*, 75(12). doi: 10.1128/IAI.00559-07.

Ansong, C. *et al.* (2009) 'Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: Implications for virulence and global protein translation', *PLoS ONE*, 4(3). doi: 10.1371/journal.pone.0004809.

Antonio Ibarra, J. *et al.* (2010) 'Induction of *Salmonella* pathogenicity island 1 under different growth conditions can affect *Salmonella*-host cell interactions in vitro', *Microbiology*, 156(4). doi: 10.1099/mic.0.032896-0.

Antunes, P. *et al.* (2011) 'Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms', *Journal of Antimicrobial Chemotherapy*, 66(9). doi: 10.1093/jac/dkr228.

Aravind, L. *et al.* (2005) 'The many faces of the helix-turn-helix domain: Transcription regulation and beyond', *FEMS Microbiology Reviews*. doi: 10.1016/j.femsre.2004.12.008.

Argüello, H. *et al.* (2018) 'Early *Salmonella* Typhimurium infection in pigs disrupts Microbiome composition and functionality principally at the ileum mucosa', *Scientific Reports*, 8(1). doi: 10.1038/s41598-018-26083-3.

Arnold, R. *et al.* (2009) 'Sequence-Based Prediction of Type III Secreted Proteins', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1000376.

Auweter, S. D. *et al.* (2011) 'Quantitative mass spectrometry catalogues *Salmonella* pathogenicity island-2 effectors and identifies their cognate host binding partners', *Journal of Biological Chemistry*, 286(27), pp. 24023–24035. doi: 10.1074/jbc.M111.224600.

Aviv, G. *et al.* (2019) 'Differences in the expression of SPI-1 genes pathogenicity and epidemiology between the emerging *Salmonella enterica* serovar Infantis and the model *Salmonella enterica* serovar Typhimurium', *Journal of Infectious Diseases*. doi: 10.1093/infdis/jiz235.

Bajaj, V. *et al.* (1996) 'Co-ordinate regulation of *Salmonella* Typhimurium invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.1996.d01-1718.x.

Bajaj, V., Hwang, C. and Lee, C. A. (1995) '*hilA* is a novel *ompR*/*toxR* family member that activates the expression of *Salmonella* Typhimurium invasion genes', *Molecualar Microbiology*, 18(4), pp. 715–727.

den Bakker, H. C. *et al.* (2011) 'Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*', *BMC Genomics*. BioMed Central Ltd, 12(1), p. 425. doi: 10.1186/1471-2164-12-425.

Bakowski, M. A. *et al.* (2007) 'SopD acts cooperatively with SopB during *Salmonella enterica* serovar Typhimurium invasion', *Cellular Microbiology*, 9(12), pp. 2839–2855. doi: 10.1111/j.1462-5822.2007.01000.x.

Bakowski, M. A. *et al.* (2010) 'The phosphoinositide phosphatase SopB manipulates membrane surface charge and trafficking of the *Salmonella*-containing vacuole', *Cell Host and Microbe*. Elsevier Ltd, 7(6), pp. 453–462. doi: 10.1016/j.chom.2010.05.011.

Bakshi, C. S. *et al.* (2000) 'Identification of SopE2, a *Salmonella* secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells', *Journal of Bacteriology*, 182(8), pp. 2341–2344. doi: 10.1128/JB.182.8.2341-2344.2000.

Baldassarre, M. *et al.* (2021) 'The Rab32/BLOC-3-dependent pathway mediates host defense against different pathogens in human macrophages', *Science Advances*, 7(3). doi: 10.1126/sciadv.abb1795.

Barrow, P. A., Simpson, J. M., *et al.* (1987) 'Contribution of *Salmonella* gallinarum large plasmid toward virulence in fowl typhoid', *Infection and Immunity*, 55(2). doi: 10.1128/iai.55.2.388-392.1987.

Barrow, P. A., Huggins, M. B., *et al.* (1987) 'Observations on the pathogenesis of experimental *Salmonella* typhimurium infection in chickens.', *Research in veterinary science*, 42(2). doi: 10.1016/s0034-5288(18)30685-4.

Barthel, M. *et al.* (2003) 'Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host', *Infection and Immunity*, 71(5). doi: 10.1128/IAI.71.5.2839-2858.2003.

Batz, M. B., Henke, E. and Kowalcyk, B. (2013) 'Long-term consequences of foodborne infections', *Infectious Disease Clinics of North America*. doi: 10.1016/j.idc.2013.05.003.

Bäumler, A. J. *et al.* (1996) 'The pef fimbrial operon of *Salmonella* typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse', *Infection and Immunity*, 64(1). doi: 10.1128/iai.64.1.61-68.1996.

Baxter, M. A. *et al.* (2003) 'HilE interacts with hilD and negatively regulates hilA transcription and expression of the *Salmonella enterica* serovar typhimurium invasive phenotype', *Infection and Immunity*. doi: 10.1128/IAI.71.3.1295-1305.2003.

Bearson, S. M. D. *et al.* (2013) 'Profiling the gastrointestinal microbiota in response to *Salmonella*: Low versus high *Salmonella* shedding in the natural porcine host', *Infection, Genetics and Evolution*, 16. doi: 10.1016/j.meegid.2013.03.022.

Beltrametti, F., Kresse, A. U. and Guzmán, C. A. (1999) 'Transcriptional regulation of the *esp* genes of enterohemorrhagic *Escherichia coli*', *Journal of Bacteriology*. doi: 10.1128/jb.181.11.3409-3418.1999.

Bernal-Bayard, J. and Ramos-Morales, F. (2009) '*Salmonella* type III secretion effector SlrP is an E3 ubiquitin ligase for mammalian thioredoxin', *Journal of Biological Chemistry*, 284(40), pp. 27587–27595. doi: 10.1074/jbc.M109.010363.

Beuzon, C. R. *et al.* (2000) '*Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA', *Embo J.*, 19(13), pp. 3235-3249. doi: 10.1093/emboj/19.13.3235.

Beuzón, C. R. *et al.* (1999) 'pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella* Typhimurium', *Molecular Microbiology*, 33(4), pp. 806–816. doi: 10.1046/j.1365-2958.1999.01527.x.

Beuzón, C. R., Salcedo, S. P. and Holden, D. W. (2002) 'Growth and killing of a *Salmonella enterica* serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines', *Microbiology*, 148(9). doi: 10.1099/00221287-148-9-2705.

Bhaskaran, S. S. and Stebbins, C. E. (2012) 'Structure of the catalytic domain of the *Salmonella* virulence factor SseI', *Acta Crystallographica Section D: Biological Crystallography*. doi: 10.1107/S0907444912039042.

Bhavsar, A. P. *et al.* (2013) 'The *Salmonella* Type III Effector SspH2 Specifically Exploits the NLR Co-chaperone Activity of SGT1 to Subvert Immunity', *PLoS Pathogens*, 9(7), pp. 1–12. doi: 10.1371/journal.ppat.1003518.

Bijlsma, J. J. E. and Groisman, E. A. (2005) 'The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2005.04668.x.

Birmingham, C. L. *et al.* (2006) 'Autophagy controls *Salmonella* infection in response to damage to the Salmonella-containing vacuole', *Journal of Biological Chemistry*, 281(16). doi: 10.1074/jbc.M509157200.

Blanc-Potard, A. B. and Groisman, E. A. (1997) 'The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival', *EMBO Journal*, 16(17). doi: 10.1093/emboj/16.17.5376.

Boddicker, J. D. and Jones, B. D. (2004) 'Lon Protease Activity Causes

Down-Regulation of *Salmonella* Pathogenicity Island 1 Invasion Gene Expression after Infection of Epithelial Cells', *Infection and Immunity*, 72(4). doi: 10.1128/IAI.72.4.2002-2013.2004.

Bogomolnaya, L. M. *et al.* (2008) '"Form variation" of the O12 antigen is critical for persistence of *Salmonella* Typhimurium in the murine intestine', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2008.06461.x.

Bolton, A. J. *et al.* (1999) 'Interaction of *Salmonella* choleraesuis, *Salmonella* dublin and *Salmonella* typhimurium with porcine and bovine terminal ileum *in vivo*', *Microbiology*, 145(9), pp. 2431–2441.

Bolton, A. J., Osborne, M. P. and Stephen, J. (2000) 'Comparative study of the invasiveness of *Salmonella* serotypes Typhimurium, Choleraesuis and Dublin for Caco-2 cells, HEp-2 cells and rabbit ileal epithelia', *Journal of Medical Microbiology*. doi: 10.1099/0022-1317-49-6-503.

Bonardi, S. (2017) '*Salmonella* in the pork production chain and its impact on human health in the European Union', *Epidemiology and Infection*, 145(8), pp. 1513–1526. doi: 10.1017/S095026881700036X.

Botteldoorn, N. *et al.* (2006) 'Real-time reverse transcription PCR for the quantification of the mntH expression of *Salmonella enterica* as a function of growth phase and phagosome-like conditions', *Journal of Microbiological Methods*. doi: 10.1016/j.mimet.2005.11.003.

Boyd, E. F. *et al.* (1993) '*Salmonella* reference collection B (SARB): Strains of 37 serovars of subspecies I', *Journal of General Microbiology*. doi: 10.1099/00221287-139-6-1125.

Boyen, F. *et al.* (2006) '*Salmonella* Typhimurium SPI-1 genes promote intestinal but not tonsillar colonization in pigs', *Microbes and Infection*. doi: 10.1016/j.micinf.2006.09.008.

Boyen, F. *et al.* (2008) 'A limited role for SsrA/B in persistent *Salmonella* Typhimurium infections in pigs', *Veterinary Microbiology*. doi: 10.1016/j.vetmic.2007.10.031.

Boyle, E. C., Brown, N. F. and Finlay, B. B. (2006) '*Salmonella enterica* serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function', *Cellular Microbiology*. doi: 10.1111/j.1462-5822.2006.00762.x.

Branchu, P., Bawn, M. and Kingsley, R. A. (2018) 'Genome variation and molecular epidemiology of *Salmonella enterica* serovar Typhimurium pathovariants', *Infection and Immunity*, 86(8). doi: 10.1128/IAI.00079-18.

Brawn, L. C., Hayward, R. D. and Koronakis, V. (2007) '*Salmonella* SPI1 Effector SipA Persists after Entry and Cooperates with a SPI2 Effector to

Regulate Phagosome Maturation and Intracellular Replication', *Cell Host and Microbe*, 1(1). doi: 10.1016/j.chom.2007.02.001.

Vander Broek, C. W. *et al.* (2015) 'Quantitative proteomic analysis of *Burkholderia pseudomallei* Bsa type III secretion system effectors using hypersecreting mutants.', *Molecular & cellular proteomics : MCP*, 14(4), pp. 905–16. doi: 10.1074/mcp.M114.044875.

Brown, R. N. *et al.* (2012) 'A Comprehensive Subcellular Proteomic Survey of *Salmonella* Grown under Phagosome-Mimicking versus Standard Laboratory Conditions', *International Journal of Proteomics*, 2012, pp. 1–12. doi: 10.1155/2012/123076.

Brumell, J. H., Goosney, D. L. and Finlay, B. B. (2002) 'SifA, a type III secreted effector of *Salmonella* Typhimurium, directs *Salmonella*-induced filament (Sif) formation along microtubules', *Traffic (Copenhagen, Denmark)*, 3(6), pp. 407–415. doi: 10.1034/j.1600-0854.2002.30604.x.

Bruno, V. M. *et al.* (2009) '*Salmonella* Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells', *PLoS Pathogens*, 5(8). doi: 10.1371/journal.ppat.1000538.

Burns-Keliher, L. *et al.* (1998) 'Cell-specific proteins synthesized by *Salmonella* Typhimurium', *Infection and Immunity*. doi: 10.1128/iai.66.2.856-861.1998.

Bustamante, V. H. *et al.* (2008) 'HilD-mediated transcriptional cross-talk between SPI-1 and SPI-2', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0801205105.

Büttner, D. (2012) 'Protein Export According to Schedule: Architecture, Assembly, and Regulation of Type III Secretion Systems from Plant- and Animal-Pathogenic Bacteria', *Microbiology and Molecular Biology Reviews*, 76(2), pp. 262–310. doi: 10.1128/MMBR.05017-11.

Caldwell, R. B. and Lattemann, C. T. (2004) 'Simple and reliable method to precipitate proteins from bacterial culture supernatant', *Applied and environmental microboology*, 70(1), pp. 610–612. doi: 10.1128/AEM.70.1.610.

Cameron, A. D. S. *et al.* (2017) 'Broad-scale redistribution of mRNA abundance and transcriptional machinery in response to growth rate in *Salmonella enterica* serovar Typhimurium', *Microbial Genomics*, 3(10). doi: 10.1099/mgen.0.000127.

Campos, J. *et al.* (2019) 'Non-typhoidal salmonella in the pig production chain: A comprehensive analysis of its impact on human health', *Pathogens*. doi: 10.3390/pathogens8010019.

Carden, S. *et al.* (2015) 'Non-typhoidal *Salmonella* Typhimurium ST313 isolates that cause bacteremia in humans stimulate less inflammasome activation than ST19 isolates associated with gastroenteritis', *Pathogens and disease*, 73(4), pp. 1–8. doi: 10.1093/femspd/ftu023.

Carden, S. E. *et al.* (2017) 'Pseudogenization of the Secreted Effector Gene sseI Confers Rapid Systemic Dissemination of *S.* Typhimurium ST313 within Migratory Dendritic Cells', *Cell Host & Microbe*. Elsevier Inc., 21(2), pp. 182–194. doi: 10.1016/j.chom.2017.01.009.

Cardenal-Muñoz, E. and Ramos-Morales, F. (2011) 'Analysis of the expression, secretion and translocation of the salmonella enterica type iii secretion system effector *stea'*, *PLoS ONE*. doi: 10.1371/journal.pone.0026930.

Carina Audisio, M. and Terzolo, H. R. (2002) 'Virulence analysis of a *Salmonella* gallinarum strain by oral inoculation of 20-day-old chickens', *Avian Diseases*, 46(1). doi: 10.1637/0005-2086(2002)046[0186:vaoasg]2.0.co;2.

Carnell, S. C. *et al.* (2007) 'Role in virulence and protective efficacy in pigs of *Salmonella enterica* serovar Typhimurium secreted components identified by signature-tagged mutagenesis', *Microbiology*, 153(6), pp. 1940–1952. doi: 10.1099/mic.0.2006/006726-0.

Carter, P. B. and Collins, F. M. (1974) 'The route of enteric infection in normal mice', *Journal of Experimental Medicine*, 139(5). doi: 10.1084/jem.139.5.1189.

Centers for Disease Control and Prevention (CDC) (2018) 'National Enteric Disease Surveillance : *Salmonella* Annual Summary , 2016', *Atlantic*, (December).

Centers for Disease Control and Prevention (CDC) (2019) *Salmonella Infections Linked to Ground Beef*.

Chadfield, M. S. *et al.* (2003) 'Comparison of intestinal invasion and macrophage response of *Salmonella* Gallinarum and other host-adapted *Salmonella enterica* serovars in the avian host', *Veterinary Microbiology*, 92(1–2), pp. 49–64. doi: 10.1016/S0378-1135(02)00290-0.

Chamaillard, M. *et al.* (2003) 'An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid', *Nature Immunology*. doi: 10.1038/ni945.

Chan, F. K.-M., Moriwaki, K. and Rosa, M. J. De (2009) 'Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) Activity', *Methods Mol Biol*, 531(1).

Chang, J. *et al.* (2007) 'SipC multimerization promotes actin nucleation and contributes to *Salmonella*-induced inflammation', *Molecular Microbiology*, 66(6), pp. 1548–1556. doi: 10.1111/j.1365-2958.2007.06024.x.

Chang, J., Chen, J. and Zhou, D. (2005) 'Delineation and characterization of the actin nucleation and effector translocation activities of *Salmonella* SipC', *Molecular Microbiology*, 55(5), pp. 1379–1389. doi: 10.1111/j.1365-2958.2004.04480.x.

Charles, S. D. *et al.* (2000) 'Reduced shedding and clinical signs of *Salmonella* Typhimurium in nursery pigs vaccinated with a *Salmonella* Choleraesuis vaccine', *Journal of Swine Health and Production*, 8(3).

Chatfield, S. N. *et al.* (1991) 'Role of *ompR*-dependent genes in *Salmonella* typhimurium virulence: Mutants deficient in both *ompC* and *ompF* are attenuated *in vivo'*, *Infection and Immunity*. doi: 10.1128/iai.59.1.449-452.1991.

Chaudhuri, R. R. *et al.* (2009) 'Comprehensive identification of *Salmonella enterica* serovar typhimurium genes required for infection of BALB/c mice', *PLoS Pathogens*, 5(7). doi: 10.1371/journal.ppat.1000529.

Chaudhuri, R. R. *et al.* (2013) 'Comprehensive Assignment of Roles for *Salmonella* Typhimurium Genes in Intestinal Colonization of Food-Producing Animals', *PLoS Genetics*, 9(4). doi: 10.1371/journal.pgen.1003456.

Chen, L. M., Hobbie, S. and Galán, J. E. (1996) 'Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses', *Science*, 274(5295). doi: 10.1126/science.274.5295.2115.

Chen, L. M., Kaniga, K. and Galán, J. E. (1996) '*Salmonella* spp. are cytotoxic for cultured macrophages.', *Molecular microbiology*, 21(5), pp. 1101–15. doi: 10.1046/j.1365-2958.1996.471410.x.

Chen, P. L. *et al.* (2007) 'Extraintestinal focal infections in adults with nontyphoid Salmonella bacteraemia: Predisposing factors and clinical outcome', in *Journal of Internal Medicine*. doi: 10.1111/j.1365-2796.2006.01748.x.

Cheng, S. *et al.* (2017) 'Identification of a novel *Salmonella* type III effector by quantitative secretome profiling', *Molecular & Cellular Proteomics*, p. mcp.000230.2017. doi: 10.1074/mcp.RA117.000230.

Cheng, W. L. *et al.* (2016) '*Salmonella* infective endocarditis', *Journal of Microbiology, Immunology and Infection*. doi: 10.1016/j.jmii.2015.02.659.

Chieppa, M. *et al.* (2006) 'Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement', *Journal of Experimental Medicine*. doi: 10.1084/jem.20061884.

Chiu, C. H., Su, L. H. and Chu, C. (2004) '*Salmonella enterica* Serotype Choleraesuis: Epidemiology, Pathogenesis, Clinical Disease, and Treatment', *Clinical Microbiology Reviews*, 17(2), pp. 311–322. doi: 10.1128/CMR.17.2.311-322.2004.

Chiu, S., Chiu, C. H. and Lin, T. Y. (2004) '*Salmonella enterica* serotype Choleraesuis infection in a medical center in northern Taiwan', *Journal of Microbiology, Immunology and Infection*, 37(2).

Cho, B.-K. *et al.* (2009) 'The transcription unit architecture of the *Escherichia coli* genome', *Nature Biotechnology*. Nature Publishing Group, 27(11), pp. 1043–1049. doi: 10.1038/nbt.1582.

Choi, K. H., Kumar, A. and Schweizer, H. P. (2006) 'A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation', *Journal of Microbiological Methods*, 64(3). doi: 10.1016/j.mimet.2005.06.001.

Chong, A. *et al.* (2019) 'A role for the *Salmonella* Type III Secretion System 1 in bacterial adaptation to the cytosol of epithelial cells', *Molecular Microbiology*. doi: 10.1111/mmi.14361.

Chu, C. *et al.* (1999) 'Comparative physical and genetic maps of the virulence plasmids of *Salmonella enterica* serovars typhimurium, enteritidis, choleraesuis, and dublin', *Infection and Immunity*. doi: 10.1128/iai.67.5.2611-2614.1999.

Cirillo, D. M. *et al.* (1998) 'Macrophage-dependent induction of the *Salmonell*a pathogenicity island 2 type III secretion system and its role in intracellular survival.', *Molecular microbiology*, 30(1), pp. 175–88. doi: 10.1046/j.1365-2958.1998.01048.x.

Clark, L. *et al.* (2011) 'Differences in *Salmonella enterica* serovar typhimurium strain invasiveness are associated with heterogeneity in SPI-1 gene expression', *Microbiology*, 157(7), pp. 2072–2083. doi: 10.1099/mic.0.048496-0.

Coburn, B., Grassl, G. A. and Finlay, B. B. (2006) '*Salmonella*, the host and disease', *Immunology and Cell Biology*, 85, pp. 112–118. doi: 10.1038/sj.icb.7100007.

Collado-Romero, M. *et al.* (2010) 'Quantitative analysis of the immune response upon *Salmonella* typhimurium infection along the porcine intestinal gut', *Veterinary Research*, 41(2). doi: 10.1051/vetres/2009072.

Conner, C. P. *et al.* (1998) 'Differential patterns of acquired virulence genes distinguish *Salmonella* strains.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(8), pp. 4641–5. doi:

10.1073/pnas.95.8.4641.

Coombes, B. K. *et al.* (2004) 'Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL', *Journal of Biological Chemistry*, 279(48), pp. 49804–49815. doi: 10.1074/jbc.M404299200.

Cooper, C. A. *et al.* (2010) 'Structural and biochemical characterization of SrcA, a multi-cargo type III secretion chaperone in *Salmonella* required for pathogenic association with a host', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1000751.

Cossart, P. and Sansonetti, P. J. (2004) 'Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens', *Science*. doi: 10.1126/science.1090124.

Criss, a K. *et al.* (2001) 'The GTPase Rac1 selectively regulates *Salmonella* invasion at the apical plasma membrane of polarized epithelial cells.', *Journal of cell science*, 114(Pt 7), pp. 1331–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11256999.

D'Costa, V. M. *et al.* (2015) '*Salmonella* Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7', *Cell Reports*, 12(9). doi: 10.1016/j.celrep.2015.07.063.

Darwin, K. H. and Miller, V. L. (1999) 'InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in *Salmonella* typhimurium', *Journal of Bacteriology*, 181(16), pp. 4949–4954. doi: 10.1128/jb.183.16.4876-4885.2001.

Darwin, K. H. and Miller, V. L. (2000) 'The putative invasion protein chaperone SicA acts together with InvF to activate the expression of *Salmonella* typhimurium virulence genes', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2000.01772.x.

Darwin, K. H. and Miller, V. L. (2001) 'Type III secretion chaperone-dependent regulation: Activation of virulence genes by SicA and InvF in *Salmonella* typhimurium', *EMBO Journal*, 20(8), pp. 1850–1862. doi: 10.1093/emboj/20.8.1850.

Davies, M. R. *et al.* (2013) 'Horizontally Acquired Glycosyltransferase Operons Drive Salmonellae Lipopolysaccharide Diversity', *PLoS Genetics*. doi: 10.1371/journal.pgen.1003568.

Davis, A. J. and Mecsas, J. (2007) 'Mutations in the *Yersinia pseudotuberculosis* type III secretion system needle protein, YscF, that specifically abrogate effector translocation into host cells', *Journal of Bacteriology*, 189(1), pp. 83–97. doi: 10.1128/JB.01396-06.

Dean, P. and Kenny, B. (2009) ‘The effector repertoire of enteropathogenic E. coli: ganging up on the host cell’, *Current Opinion in Microbiology*. doi: 10.1016/j.mib.2008.11.006.

Deiwick, J. *et al.* (1999) ‘Environmental regulation of *Salmonella* pathogenicity island 2 gene expression.’, *Molecular microbiology*, 31(6), pp. 1759–1773. doi: 10.1046/j.1365-2958.1999.01312.x.

Deiwick, J. *et al.* (2006) ‘The translocated *Salmonella* effector proteins SseF and SseG interact and are required to establish an intracellular replication niche’, *Infection and Immunity*, 74(12), pp. 6965–6972. doi: 10.1128/IAI.00648-06.

Deng, X. *et al.* (2014) ‘Genomic epidemiology of *Salmonella enterica* serotype Enteritidis based on population structure of prevalent lineages’, *Emerging Infectious Diseases*, 20(9). doi: 10.3201/eid2009.131095.

Didelot, X. *et al.* (2011) ‘Recombination and population structure in *Salmonella enterica*’, *PLoS Genetics*, 7(7). doi: 10.1371/journal.pgen.1002191.

Diepold, A. and Armitage, J. P. (2015) ‘Type III secretion systems: the bacterial flagellum and the injectisome’, *Philosophical Transactions of the Royal Society B*, 370(1679), p. 20150020. doi: 10.1098/rstb.2015.0020.

Diepold, A. and Wagner, S. (2014) ‘Assembly of the bacterial type III secretion machinery’, *FEMS Microbiology Reviews*. doi: 10.1111/1574-6976.12061.

Domingues, L., Holden, D. W. and Mota, L. J. (2014) ‘The *Salmonella* effector SteA contributes to the control of membrane dynamics of *Salmonella*-containing vacuoles’, *Infection and Immunity*, 82(7), pp. 2923–2934. doi: 10.1128/IAI.01385-13.

Dorman, C. J. (2007) ‘H-NS, the genome sentinel’, *Nature Reviews Microbiology*. doi: 10.1038/nrmicro1598.

Dougan, G. and Baker, S. (2014) ‘*Salmonella enterica* serovar typhi and the pathogenesis of typhoid fever’, *Annual Review of Microbiology*. doi: 10.1146/annurev-micro-091313-103739.

Drecktrah, D. *et al.* (2007) ‘*Salmonella* trafficking is defined by continuous dynamic interactions with the endolysosomal system’, *Traffic*, 8(3). doi: 10.1111/j.1600-0854.2006.00529.x.

Drumo, R. *et al.* (2016) ‘*Salmonella enterica* serovar typhimurium exploits inflammation to modify swine intestinal microbiota’, *Frontiers in Cellular and Infection Microbiology*, 5(JAN). doi: 10.3389/fcimb.2015.00106.

Dubey, A. K. *et al.* (2005) 'RNA sequence and secondary structure participate in high-affinity CsrA-RNA interaction', *RNA*. doi: 10.1261/rna.2990205.

Dukes, J. D. *et al.* (2006) 'The secreted *Salmonella* dublin phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking', *Biochemical Journal*, 395(2), pp. 239–247. doi: 10.1042/BJ20051451.

Duong, N. *et al.* (2007) 'Thermosensing coordinates a cis-regulatory module for transcriptional activation of the intracellular virulence system in *Salmonella enterica* serovar typhimurium', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M707352200.

Eberth, C. J. (1880) 'Die Organismen in den Organen bei Typhus abdominalis', *Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin*. doi: 10.1007/BF01995472.

Eddicks, M. *et al.* (2009) 'Examination of the compatibility of a *Salmonella* typhimurium-live vaccine salmoporc® for three day old suckling piglets', *DTW. Deutsche Tierärztliche Wochenschrift*, 116(7). doi: 10.2376/0341-6593-116-249.

EFSA (2018) 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017', *EFSA Journal*, 16(12). doi: 10.2903/j.efsa.2018.5500.

EFSA (2019) 'The European Union One Health 2018 Zoonoses Report', *EFSA Journal*. doi: 10.2903/j.efsa.2019.5926.

Eichinger, V. *et al.* (2016) 'EffectiveDB - Updates and novel features for a better annotation of bacterial secreted proteins and Type III, IV, VI secretion systems', *Nucleic Acids Research*, 44(D1), pp. D669–D674. doi: 10.1093/nar/gkv1269.

Elhadad, D. *et al.* (2015) 'Flagellin is required for host cell invasion and normal *Salmonella* pathogenicity island 1 expression by *Salmonella enterica* serovar Paratyphi A', *Infection and Immunity*, 83(9), pp. 3355–3368. doi: 10.1128/IAI.00468-15.

Elhadad, D., Desai, P., Grassl, Guntram A., *et al.* (2016) 'Differences in host cell invasion and *Salmonella* pathogenicity island 1 expression between *Salmonella enterica* serovar Paratyphi A and nontyphoidal *S.* Typhimurium', *Infection and Immunity*, 84(4), pp. 1150–1165. doi: 10.1128/IAI.01461-15.

Ellermeier, C. D., Ellermeier, J. R. and Slauch, J. M. (2005) 'HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator *hilA* in *Salmonella enterica* serovar Typhimurium', *Molecular Microbiology*. doi: 10.1111/j.1365-

2958.2005.04737.x.

Erhardt, M. and Dersch, P. (2015) ‘Regulatory principles governing *Salmonella* and *Yersinia* virulence’, *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.00949.

Eriksson, S. *et al.* (2003) ‘Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*’, *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2003.03313.x.

European Food Safety Authority European Centre for Disease Prevention and Control (2018) *Multi-country outbreak of Salmonella Agona infections possibly linked to ready-to-eat food*.

Faber, F. *et al.* (2017) ‘Respiration of Microbiota-Derived 1,2-propanediol Drives *Salmonella* Expansion during Colitis’, *PLoS Pathogens*, 13(1), pp. 1–19. doi: 10.1371/journal.ppat.1006129.

Faucher, S. P., Curtiss, R. and Daigle, F. (2005) ‘Selective capture of Salmonella enterica serovar Typhi genes expressed in macrophages that are absent from the *Salmonella enterica* serovar Typhimurium genome’, *Infection and Immunity*, 73(8). doi: 10.1128/IAI.73.8.5217-5221.2005.

Feasey, N. A. *et al.* (2012) ‘Invasive non-typhoidal salmonella disease: An emerging and neglected tropical disease in Africa’, *The Lancet*. doi: 10.1016/S0140-6736(11)61752-2.

Ferstl, P. G. *et al.* (2017) ‘Severe infection with multidrug-resistant *Salmonella choleraesuis* in a young patient with primary sclerosing cholangitis’, *World Journal of Gastroenterology*, 23(11), p. 2086. doi: 10.3748/wjg.v23.i11.2086.

Fields, P. I. *et al.* (1986) ‘Mutants of *Salmonella* typhimurium that cannot survive within the macrophage are avirulent’, *Proceedings of the Nationall Academy of Sciences of the United States of America*, 83(14), pp. 5189–5193. doi: 10.1073/pnas.83.14.5189.

Fierer, J. and Guiney, D. G. (2001) ‘Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection’, *Journal of Clinical Investigation*, 107(7), pp. 775–780. doi: 10.1172/JCI12561.

Figueiredo, J. F. *et al.* (2009) ‘*Salmonella* enterica Typhimurium SipA induces CXC-chemokine expression through p38MAPK and JUN pathways’, *Microbes and Infection*, 11(2), pp. 302–310. doi: 10.1016/j.micinf.2008.12.005.

Fink, S. L. and Cookson, B. T. (2006) ‘Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages’, *Journal of Immunology*, 202(7). doi: 10.1111/J.1462-5822.2006.00751.x.

Finn, C. E. *et al.* (2017) 'A second wave of *Salmonella* T3SS1 activity prolongs the lifespan of infected epithelial cells', *PLOS Pathogens*, 13(4), p. e1006354. doi: 10.1371/journal.ppat.1006354.

Foley, S. L. *et al.* (2013) '*Salmonella* pathogenicity and host adaptation in chicken-associated serovars.', *Microbiology and molecular biology reviews : MMBR*, 77(4), pp. 582–607. doi: 10.1128/MMBR.00015-13.

Foster, J. W. and Hall, H. K. (1990) 'Adaptive acidification tolerance response of *Salmonella* typhimurium', *Journal of Bacteriology*, 172(2). doi: 10.1128/jb.172.2.771-778.1990.

Fredlund, J. *et al.* (2017) 'The entry of *Salmonella* in a distinct tight compartment revealed at high temporal and ultrastructural resolution', *Cellular Microbiology*, 1, p. e12816. doi: 10.1111/cmi.12816.

Friebel, A. *et al.* (2001) 'SopE and SopE2 from *Salmonella* typhimurium Activate Different Sets of RhoGTPases of the Host Cell', *Journal of Biological Chemistry*, 276(36), pp. 34035–34040. doi: 10.1074/jbc.M100609200.

Friesema, I. *et al.* (2014) 'Large outbreak of *Salmonella* Tompson related to smoked salmon in the Netherlands, August to December 2012', *Eurosurveillance*, 19(39). doi: 10.2807/1560-7917.es2014.19.39.20918.

Frost, A. ., Bland, A. . and Wallis, T. . (1997) 'The Early Dynamic Response of the Calf Ileal Epithelium to *Salmonella* typhimurium', *Veterinary Pathology*, 34, pp. 369–386.

Fu, Y. and Galán, J. E. (1999) 'A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion.', *Nature*, 401(6750), pp. 293–7. doi: 10.1038/45829.

Gagic, D. *et al.* (2016) 'Exploring the secretomes of microbes and microbial communities using filamentous phage display', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00429.

Gal-Mor, O. *et al.* (2008) 'A novel secretion pathway of *Salmonella enterica* acts as an antivirulence modulator during salmonellosis', *PLoS Pathogens*, 4(4). doi: 10.1371/journal.ppat.1000036.

Gal-Mor, O. *et al.* (2011) 'The *Salmonella enterica* PhoP directly activates the horizontally acquired SPI-2 gene *ssel* and is functionally different from a *S. bongori* ortholog', *PLoS ONE*, 6(5). doi: 10.1371/journal.pone.0020024.

Galán, J. E. *et al.* (2014) 'Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells.', *Annual review of microbiology*, 68, pp. 415–38. doi: 10.1146/annurev-micro-092412-155725.

Galan, J. E. and Collmer, A. (1999) 'Type III Secretion Machines: Bacterial

Devices for Protein Delivery into Host Cells', *Science*, 284(5418), pp. 1322–1329. doi: 10.1126/science.284.5418.1322.

Galan, J. E. and Wolf-watz, H. (2006) 'Protein delivery into eukaryotic cells by type III secretion machines', *Nature*, 444(7119), pp. 567–573. doi: 10.1038/nature05272.

Galyov, E. E. *et al.* (1997) 'A secreted effector protein of *Salmonella* dublin is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa.', *Molecular microbiology*, 25(5), pp. 903–12. doi: 10.1111/j.1365-2958.1997.mmi525.x.

Garcia-del Portillo, F. *et al.* (1993) '*Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells', *Proceedings of the National Academy of Sciences of the United States of America*, 90(22). doi: 10.1073/pnas.90.22.10544.

Garcia-Del Portillo, F. and Finlay, B. B. (1995) 'Targeting of *Salmonella* typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors', *Journal of Cell Biology*, 129(1). doi: 10.1083/jcb.129.1.81.

Garmendia, J. *et al.* (2003) 'The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella* typhimurium SPI-2 type III secretion system', *Microbiology*, 149(9), pp. 2385–2396. doi: 10.1099/mic.0.26397-0.

Garmendia, J. *et al.* (2004) 'TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton', *Cellular Microbiology*, 6(12). doi: 10.1111/j.1462-5822.2004.00459.x.

Gawande, P. V. and Bhagwat, A. A. (2002) 'Inoculation onto solid surfaces protects *Salmonella* spp. during acid challenge: A model study using polyethersulfone membranes', *Applied and Environmental Microbiology*, 68(1). doi: 10.1128/AEM.68.1.86-92.2002.

Geddes, K. *et al.* (2005) 'Identification of New Secreted Effectors', *Infection and immunity*, 73(10), pp. 6260–6271. doi: 10.1128/IAI.73.10.6260.

Gerlach, R. G. *et al.* (2007) '*Salmonella* pathogenicity island 4-mediated adhesion is coregulated with invasion genes in *Salmonella enterica*', *Infection and Immunity*, 75(10), pp. 4697–4709. doi: 10.1128/IAI.00228-07.

Gerlach, R. G. *et al.* (2008) 'Cooperation of *Salmonella* pathogenicity islands 1 and 4 is required to breach epithelial barriers', *Cellular Microbiology*, 10(11), pp. 2364–2376. doi: 10.1111/j.1462-5822.2008.01218.x.

Gewirtz, A. T. *et al.* (2000) '*Salmonella* typhimurium induces epithelial IL-8 expression via Ca2+- mediated activation of the NF-κb pathway', *Journal of*

Clinical Investigation, 105(1). doi: 10.1172/JCI8066.

Gewirtz, A. T. *et al.* (2001) '*Salmonella* typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response', *Journal of Clinical Investigation*, 107(1). doi: 10.1172/JCI10501.

Giannella, R. A. *et al.* (1973) 'Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum.', *The Journal of clinical investigation*. doi: 10.1172/JCI107201.

Ginestet, C. (2011) 'ggplot2: Elegant Graphics for Data Analysis', *Journal of the Royal Statistical Society: Series A (Statistics in Society)*, 174(1). doi: 10.1111/j.1467-985x.2010.00676_9.x.

Godlee, C. *et al.* (2018) 'SrcA is a chaperone for the *Salmonella* SPI-2 type three secretion system effector SteD', *Microbiology*, pp. 1–11. doi: 10.1099/mic.0.000732.

Golubeva, Y. A. *et al.* (2012) 'Integrating global regulatory input into the *Salmonella* pathogenicity Island 1 type III secretion system', *Genetics*. doi: 10.1534/genetics.111.132779.

Gomes-Neves, E. *et al.* (2014) 'Clinically relevant multidrug resistant *Salmonella enterica* in swine and meat handlers at the abattoir', *Veterinary Microbiology*, 168(1). doi: 10.1016/j.vetmic.2013.10.017.

Gomez-Valero, L. *et al.* (2019) 'More than 18,000 effectors in the *Legionella* genus genome provide multiple, independent combinations for replication in human cells', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1808016116.

Gong, H. *et al.* (2009) 'Characterization of the expression of *Salmonella* Type III secretion system factor PrgI, SipA, SipB, SopE2, SpaO, and SptP in cultures and in mice', *BMC Microbiology*. doi: 10.1186/1471-2180-9-73.

Gong, H. *et al.* (2010) 'Differential expression of *Salmonella* type III secretion system factors InvJ, PrgJ, SipC, SipD, SopA and SopB in cultures and in mice', *Microbiology*, 156(1), pp. 116–127. doi: 10.1099/mic.0.032318-0.

Gong, L. *et al.* (2011) 'The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis', *PLoS ONE*, 6(3). doi: 10.1371/journal.pone.0017852.

Gopinath, A. *et al.* (2019) 'The *Salmonella* type III effector SpvC triggers the reverse transmigration of infected cells into the bloodstream', *PLoS ONE*. doi: 10.1371/journal.pone.0226126.

Gouw, M. *et al.* (2018) 'The eukaryotic linear motif resource - 2018 update', *Nucleic Acids Research*, 46(D1), pp. D428–D434. doi: 10.1093/nar/gkx1077.

Gragg, S. E. *et al.* (2013) 'Substantial within-animal diversity of *salmonella* isolates from lymph nodes, feces, and hides of cattle at slaughter', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01020-13.

Grenz, J. R. *et al.* (2018) 'HilE regulates HilD by blocking DNA binding in *Salmonella enterica* serovar Typhimurium', *Journal of Bacteriology*, 200(8), pp. 1–13. doi: 10.1055/s-2003-814849.

Groisman, E. A. (2001) 'The pleiotropic two-component regulatory system PhoP-PhoQ', *Journal of Bacteriology*. doi: 10.1128/JB.183.6.1835-1842.2001.

Groisman, E. A. and Ochman, H. (1996) 'Pathogenicity islands: Bacterial evolution in quantum leaps', *Cell*, 87(5), pp. 791–794. doi: 10.1016/S0092-8674(00)81985-6.

Groninga, K. J. *et al.* (2000) '*Salmonella* derby cross-protection study.', *Veterinary therapeutics : research in applied veterinary medicine*, 1(1).

Guiney, D. G. *et al.* (1994) 'Plasmid-mediated virulence genes in non-typhoid *Salmonella* serovars', *FEMS Microbiology Letters*. doi: 10.1111/j.1574-6968.1994.tb07253.x.

Guiney, D. G. *et al.* (1995) 'Biology and clinical significance of virulence plasmids in *Salmonella* serovars', *Clinical Infectious Diseases*. doi: 10.1093/clinids/21.Supplement_2.S146.

Gulig, P. A. *et al.* (1997) 'Systemic infection of mice by wild-type but not Spv-*Salmonella* typhimurium is enhanced by neutralization of gamma interferon and tumor necrosis factor alpha', *Infection and Immunity*. doi: 10.1128/iai.65.12.5191-5197.1997.

Gulig, P. A. and Doyle, T. J. (1993) 'The *Salmonella* typhimurium virulence plasmid increases the growth rate of salmonellae in mice', *Infection and Immunity*. doi: 10.1128/iai.61.2.504-511.1993.

Günzel, D. *et al.* (2006) 'The MgtC virulence factor of *Salmonella enterica* serovar typhimurium activates Na+,K+-ATPase', *Journal of Bacteriology*, 188(15). doi: 10.1128/JB.00296-06.

Habyarimana, F., Sabag-Daigle, A. and Ahmer, B. M. M. (2014) 'The SdiA-regulated gene *srgE* encodes a type III secreted effector', *Journal of Bacteriology*, 196(12). doi: 10.1128/JB.01602-14.

Haghjoo, E. and Galán, J. E. (2004) '*Salmonella* typhi encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway', *Proceedings of the National Academy of Sciences of the United States of America*, 101(13). doi: 10.1073/pnas.0400932101.

Handler, D. C. *et al.* (2018) 'The Art of Validating Quantitative Proteomics Data', *Proteomics*, 18(23). doi: 10.1002/pmic.201800222.

Haneda, T. *et al.* (2012) '*Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection', *Cellular Microbiology*, 14(4). doi: 10.1111/j.1462-5822.2011.01733.x.

Hanna, J., Ellis, W. A. and O'Brien, J. J. (1979) 'Immunisation of pregnant sows with a live *Salmonella* choleraesuis vaccine', *Veterinary Microbiology*, 3(4). doi: 10.1016/0378-1135(79)90006-3.

Hanna, J., McCracken, R. and O'Brien, J. J. (1979) 'Evaluation of a live *Salmonella* choleraesuis vaccine by intranasal challenge', *Research in Veterinary Science*, 26(2). doi: 10.1016/s0034-5288(18)32920-5.

Hardt, W. D. *et al.* (1998) '*S.* typhimurium Encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells', *Cell*, 93(5), pp. 815–826. doi: 10.1016/S0092-8674(00)81442-7.

Hardt, W. D., Urlaub, H. and Galán, J. E. (1998) 'A substrate of the centisome 63 type III protein secretion system of *Salmonella* typhimurium is encoded by a cryptic bacteriophage', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.95.5.2574.

Harris, M. (1960) 'Specificity and cross-reactions in a strain of pig kidney cells resistant to 2,6-diaminopurine', *Experimental Cell Research*, 21(2). doi: 10.1016/0014-4827(60)90276-7.

Harvey, R. R. *et al.* (2017) 'Epidemiology of *Salmonella enterica* serotype Dublin infections among humans, United States, 1968–2013', *Emerging Infectious Diseases*, 23(9). doi: 10.3201/eid2309.170136.

Hashimoto-Gotoh, T. *et al.* (1981) 'Specific-purpose plasmid cloning vectors I. Low copy number, temperature-sensitive, mobilization-defective pSC101-derived containment vectors', *Gene*. doi: 10.1016/0378-1119(81)90079-2.

Hausmann, A. *et al.* (2020) 'Intestinal epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen *Salmonella* Typhimurium due to site-specific bacterial PAMP expression.', *Mucosal immunology*. Springer US, (November 2019). doi: 10.1038/s41385-019-0247-0.

Hautefort, I. *et al.* (2003) 'Single-Copy Green Fluorescent Protein Gene Fusions Allow Accurate Measurement of *Salmonella* Gene Expression In Vitro and during Infection of Mammalian Cells', *Applied and Environmental Microbiology*, 69(12), pp. 7480–7491. doi: 10.1128/AEM.69.12.7480.

Hautefort, I. *et al.* (2008) 'During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional

adaptation that results in simultaneous expression of three type 3 secretion systems', *Cellular Microbiology*, 10(4). doi: 10.1111/j.1462-5822.2007.01099.x.

Havelaar, A. H. *et al.* (2015) 'World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010', *PLoS Medicine*, 12(12), pp. 1–23. doi: 10.1371/journal.pmed.1001923.

Hayashi, F. *et al.* (2001) 'The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5', *Nature*. doi: 10.1038/35074106.

Hayward, R. D. and Koronakis, V. (1999) 'Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella'*, *EMBO Journal*, 18(18), pp. 4926–4934. doi: 10.1093/emboj/18.18.4926.

Hébrard, M. *et al.* (2011) 'The challenge of relating gene expression to the virulence of *Salmonella enterica* serovar Typhimurium', *Current Opinion in Biotechnology*. Elsevier Ltd, 22(2), pp. 200–210. doi: 10.1016/j.copbio.2011.02.007.

Helaine, S. *et al.* (2010) 'Dynamics of intracellular bacterial replication at the single cell level', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1000041107.

Hensel, M. *et al.* (1998) 'Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages', *Molecular Microbiology*, 30, pp. 163–174.

Hensel, M. and et al. (1995) 'Simultaneous identification of bacterial virulence genes by negative selection', *Science 269:400-403*, 269(5222), pp. 400–403.

Hernandez, L. D. *et al.* (2004) '*Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism', *Science*. doi: 10.1126/science.1098188.

Hersh, D. *et al.* (1999) 'The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(5), pp. 2396–401. doi: 10.1073/pnas.96.5.2396.

Higashide, W. and Zhou, D. (2006) 'The first 45 amino acids of SopA are necessary for InvB binding and SPI-1 secretion', *Journal of Bacteriology*, 188(7), pp. 2411–2420. doi: 10.1128/JB.188.7.2411-2420.2006.

Hobbie, S. *et al.* (1997) 'Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella* typhimurium in cultured intestinal epithelial cells.', *Journal of immunology (Baltimore, Md. : 1950)*, 159(11).

Hobbs, C. K. *et al.* (2016) 'Computational approach to predict species-specific type III secretion system (T3SS) effectors using single and multiple genomes', *BMC Genomics*. BMC Genomics, 17(1), pp. 1–11. doi: 10.1186/s12864-016-3363-1.

Hoe, N. P. and Goguen, J. D. (1993) 'Temperature sensing in *Yersinia pestis*: Translation of the LcrF activator protein is thermally regulated', *Journal of Bacteriology*. doi: 10.1128/jb.175.24.7901-7909.1993.

Hoffmann, C. *et al.* (2010) 'In macrophages, caspase-1 activation by SopE and the type III secretion system-1 of *S*. Typhimurium can proceed in the absence of flagellin', *PLoS ONE*. doi: 10.1371/journal.pone.0012477.

Hoiseth, S. K. and Stocker, B. A. D. (1981) 'Aromatic-dependent *Salmonella* typhimurium are non-virulent and effective as live vaccines.', *Nature*, pp. 238–239. doi: 10.1038/291238a0.

Holmqvist, E. *et al.* (2016) ' Global RNA recognition patterns of post-transcriptional regulators Hfq and CsrA revealed by UV crosslinking in vivo ', *The EMBO Journal*. doi: 10.15252/embj.201593360.

Homma, M., Kutsukake, K. and Iino, T. (1985) 'Structural genes for flagellar hook-associated proteins in *Salmonella* typhimurium', *Journal of Bacteriology*. doi: 10.1128/jb.163.2.464-471.1985.

House, D. *et al.* (2001) 'Serology of typhoid fever in an area of endemicity and its relevance to diagnosis', *Journal of Clinical Microbiology*, 39(3). doi: 10.1128/JCM.39.3.1002-1007.2001.

Hsueh, P. R. *et al.* (2004) 'Ciprofloxacin-resistant *Salmonella enterica* Typhimurium and Choleraesuis from Pigs to Humans, Taiwan', *Emerging Infectious Diseases*. doi: 10.3201/eid1001.030171.

Hu, B., Lara-tejero, M. and Kong, Q. (2017) '*In Situ* Molecular Architecture of the *Salmonella* Type III Secretion Machine: Cell', *Cell*, 168, pp. 1065–1074. doi: 10.1016/j.cell.2017.02.022.

Huang, F. C. *et al.* (2004) 'Cooperative interactions between flagellin and SopE2 in the epithelial interleukin-8 response to *Salmonella enterica* serovar typhimurium infection', *Infection and Immunity*. doi: 10.1128/IAI.72.9.5052-5062.2004.

Huang, K. Y. *et al.* (2016) 'Hyperinvasiveness of *Salmonella enterica* serovar Choleraesuis linked to hyperexpression of type III secretion systems in vitro', *Scientific Reports*. doi: 10.1038/srep37642.

Hui, X. *et al.* (2020) 'T3SEpp: an Integrated Prediction Pipeline for Bacterial Type III Secreted Effectors', *mSystems*. doi: 10.1128/msystems.00288-20.

Hurme, R. and Rhen, M. (1998) ‘Temperature sensing in bacterial gene regulation - What it all boils down to’, *Molecular Microbiology*. doi: 10.1046/j.1365-2958.1998.01049.x.

Husa, J. A. *et al.* (2009) ‘A comparison of the safety, cross-protection, and serologic response associated with two commercial oral *Salmonella* vaccines in swine’, *Journal of Swine Health and Production*, 17(1).

Jantsch, J. *et al.* (2003) ‘Intracellular activities of *Salmonella enterica* in murine dendritic cells’, *Cellular Microbiology*. doi: 10.1046/j.1462-5822.2003.00334.x.

Jennings, E., Thurston, T. L. M. and Holden, D. W. (2017) ‘*Salmonella* SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences’, *Cell Host and Microbe*. doi: 10.1016/j.chom.2017.07.009.

Jiang, X. *et al.* (2004) ‘The related effector proteins SopD and SopD2 from *Salmonella enterica* serovar Typhimurium contribute to virulence during systemic infection of mice’, *Molecular Microbiology*, 54(5), pp. 1186–1198. doi: 10.1111/j.1365-2958.2004.04344.x.

Jneid, B. *et al.* (2020) ‘SipD and IpaD induce a cross-protection against *Shigella* and *Salmonella* infections’, *PLoS Neglected Tropical Diseases*. doi: 10.1371/journal.pntd.0008326.

Johnson, R., Ravenhall, M., *et al.* (2017) ‘Comparison of *Salmonella enterica* serovars Typhi and Typhimurium reveals typhoidal-specific responses to bile’, *Infection and Immunity*, (December), p. IAI.00490-17. doi: 10.1128/IAI.00490-17.

Johnson, R., Byrne, A., *et al.* (2017) ‘The Type III Secretion System Effector SptP of *Salmonella enterica* serovar Typhi’, *Journal of Bacteriology*, 199(4), pp. 1–18.

Johnson, R. *et al.* (2018) ‘Comparison of *Salmonella enterica* serovars Typhi and Typhimurium reveals typhoidal serovar-specific responses to bile’, *Infection and Immunity*, 86(3), pp. 1–16. doi: 10.1128/IAI.00490-17.

Jones, B. D., Ghori, N. and Falkow, S. (1994) ‘*Salmonella* typhlrnurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the peyer’s patches’, *Journal of Experimental Medicine*, 180(1). doi: 10.1084/jem.180.1.15.

Jones, C. H. *et al.* (1995) ‘FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae’, *Proceedings of the National Academy of Sciences of the United States of America*, 92(6). doi: 10.1073/pnas.92.6.2081.

Jones, M. A. *et al.* (1998) 'Secreted effector proteins of *Salmonella* dublin act in concert to induce enteritis', *Infection and Immunity*, 66(12), pp. 5799–5804.

Jones, P. W. *et al.* (1991) 'Oral vaccination of calves against experimental salmonellosis using a double aro mutant of *Salmonella* typhimurium', *Vaccine*. doi: 10.1016/0264-410X(91)90313-U.

Jones, R. M. *et al.* (2008) '*Salmonella* AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade', *Cell Host and Microbe*. doi: 10.1016/j.chom.2008.02.016.

Jones, T. *et al.* (2008) 'Salmonellosis outcomes differ substantially by serotype', *Journal of Infectious Diseases*, 198(1). doi: 10.1086/588823.

Kamanova, J. *et al.* (2016) 'The *Salmonella* Effector Protein SopA Modulates Innate Immune Responses by Targeting TRIM E3 Ligase Family Members', *PLoS Pathogens*, 12(4), pp. 1–21. doi: 10.1371/journal.ppat.1005552.

Kaniga, K. *et al.* (1995) 'Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella* typhimurium entry into cultured epithelial cells', *Journal of Bacteriology*, 177(14), pp. 3965–3971.

Karavolos, M. H. *et al.* (2005) 'Type III Secretion of the Salmonella Effector Protein SopE Is Mediated via an N-Terminal Amino Acid Signal and Not an mRNA Sequence Type III Secretion of the *Salmonella* Effector Protein SopE Is Mediated via an N-Terminal Amino Acid Signal and Not an mRNA ', *Journal of Bacteriology*, 187(5), pp. 1559–1567. doi: 10.1128/JB.187.5.1559.

Kato, J. *et al.* (2018) 'A protein secreted by the *salmonella* type iii secretion system controls needle filament assembly', *eLife*, 7, pp. 1–23. doi: 10.7554/eLife.35886.

Kawano, M., Aravind, L. and Storz, G. (2007) 'An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2007.05688.x.

Kerrinnes, T. *et al.* (2009) 'CsrA and CsrB are required for the post-transcriptional control of the virulence-associated effector protein AvrA of *Salmonella enterica*', *International Journal of Medical Microbiology*. doi: 10.1016/j.ijmm.2008.09.001.

Kikuvi, G. M., Ombui, J. N. and Mitema, E. S. (2010) 'Serotypes and antimicrobial resistance profiles of *Salmonella* isolates from pigs at slaughter in Kenya', *Journal of Infection in Developing Countries*, 4(4). doi: 10.3855/jidc.446.

Kim, K. *et al.* (2019) 'Oxygen-dependent regulation of SPI1 type three secretion system by small RNAs in *Salmonella enterica* serovar Typhimurium', *Molecular Microbiology*. doi: 10.1111/mmi.14174.

Kim, Y.-G., Kim, J.-H. and Kim, K.-J. (2009) ‘Crystal structure of the *Salmonella enterica* serovar typhimurium virulence factor SrfJ, a glycoside hydrolase family enzyme.’, *Journal of bacteriology*, 191(21), pp. 6550–4. doi: 10.1128/JB.00641-09.

Kingsley, R. A. and Bäumler, A. J. (2000) ‘Host adaptation and the emergence of infectious disease: The *Salmonella* paradigm’, *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2000.01907.x.

Kirkwood, M. *et al.* (2021) ‘Ecological niche adaptation of *Salmonella* Typhimurium U288 is associated with altered pathogenicity and reduced zoonotic potential’, *Communications Biology*, 4(498).

Kitagawa, R. *et al.* (2010) ‘Biogenesis of *Salmonella enterica* serovar typhimurium membrane vesicles provoked by induction of PagC’, *Journal of Bacteriology*. doi: 10.1128/JB.00590-10.

Klein, J. A. *et al.* (2017) ‘Controlled activity of the *Salmonella* invasion-associated injectisome reveals its intracellular role in the cytosolic population’, *mBio*, 8(6). doi: 10.1128/mBio.01931-17.

De Knegt, L. V., Pires, S. M. and Hald, T. (2015) ‘Attributing foodborne salmonellosis in humans to animal reservoirs in the European Union using a multi-country stochastic model’, *Epidemiology and Infection*, 143(6). doi: 10.1017/S0950268814001903.

Knodler, L. A. *et al.* (2002) ‘*Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems’, *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2002.02820.x.

Knodler, L. A. *et al.* (2010) ‘Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia’, *Proceedings of the National Academy of Sciences of the United States of America*, 107(41). doi: 10.1073/pnas.1006098107.

Knodler, L. A. *et al.* (2014) ‘Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens’, *Cell Host and Microbe*, 16(2). doi: 10.1016/j.chom.2014.07.002.

Knodler, L. A. (2015) ‘*Salmonella enterica*: Living a double life in epithelial cells’, *Current Opinion in Microbiology*. doi: 10.1016/j.mib.2014.10.010.

Knodler, L. A., Nair, V. and Steele-Mortimer, O. (2014) ‘Quantitative assessment of cytosolic *Salmonella* in epithelial cells’, *PLoS ONE*. doi: 10.1371/journal.pone.0084681.

Knodler, L. A. and Steele-Mortimer, O. (2005) ‘The *Salmonella* effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension.’,

Molecular biology of the cell, 16(9), pp. 4108–23. doi: 10.1091/mbc.E05-04-0367.

Köhler, H. *et al.* (2007) '*Salmonella enterica* serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage.', *American journal of physiology. Gastrointestinal and liver physiology*, 293(1), pp. G178–G187. doi: 10.1152/ajpgi.00535.2006.

Korshunov, S. S. and Imlay, J. A. (2002) 'A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria', *Molecular Microbiology*, 43(1). doi: 10.1046/j.1365-2958.2002.02719.x.

Kröger, C. *et al.* (2012) 'The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium', *Proceedings of the National Academy of Sciences*, 109(20), pp. E1277–E1286. doi: 10.1073/pnas.1201061109.

Kröger, C. *et al.* (2013) 'An infection-relevant transcriptomic compendium for *Salmonella enterica* serovar typhimurium', *Cell Host and Microbe*, 14(6), pp. 683–695. doi: 10.1016/j.chom.2013.11.010.

Kubori, T. *et al.* (1998) 'Supramolecular structure of the *Salmonella* typhimurium type III protein secretion system', *Science*. doi: 10.1126/science.280.5363.602.

Kubori, T. and Galán, J. E. (2002) '*Salmonella* type III secretion-associated protein InvE controls translocation of effector proteins into host cells', *Journal of Bacteriology*, 184(17), pp. 4699–4708. doi: 10.1128/JB.184.17.4699-4708.2002.

Kuhle, V., Jäckel, D. and Hensel, M. (2004) 'Effector proteins encoded by *Salmonella* Pathogenicity Island 2 interfere with the microtubule cytoskeleton after translocation into host cells', *Traffic*, 5(5), pp. 356–370. doi: 10.1111/j.1398-9219.2004.00179.x.

Kutsukake, K. and Ide, N. (1995) 'Transcriptional analysis of the *flgK* and *fliD* operons of *Salmonella* typhimurium which encode flagellar hook-associated proteins', *MGG Molecular & General Genetics*. doi: 10.1007/BF00293195.

Kutsukake, K. and Iino, T. (1980) 'A trans-acting factor mediates inversion of a specific DNA segment in flagellar phase variation of *Salmonella'*, *Nature*, 284(5755). doi: 10.1038/284479a0.

Lahiri, C. *et al.* (2014) 'Interactome analyses of *Salmonella* pathogenicity islands reveal SicA indispensable for virulence', *Journal of Theoretical Biology*. doi: 10.1016/j.jtbi.2014.08.013.

Lambert, M. A. and Smith, S. G. J. (2008) 'The PagN protein of *Salmonella*

enterica serovar Typhimurium is an adhesin and invasin', *BMC Microbiology*. doi: 10.1186/1471-2180-8-142.

Langridge, G. C. *et al.* (2015) 'Patterns of genome evolution that have accompanied host adaptation in *Salmonella*.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(3), pp. 863–8. doi: 10.1073/pnas.1416707112.

Lara-Tejero, M. *et al.* (2011) 'A sorting platform determines the order of protein secretion in bacterial type III systems.', *Science (New York, N.Y.)*, 331(6021), pp. 1188–91. doi: 10.1126/science.1201476.

Lara-Tejero, M. and Galán, J. E. (2009) '*Salmonella enterica* serovar Typhimurium pathogenicity island 1-encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells', *Infection and Immunity*, 77(7), pp. 2635–2642. doi: 10.1128/IAI.00077-09.

Lawhon, S. D. *et al.* (2003) 'Global regulation by CsrA in *Salmonella* typhimurium', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2003.03535.x.

Lee, A. K., Detweiler, C. S. and Falkow, S. (2000) 'OmpR regulates the two-component system SsrA-SsrB in Salmonella pathogenicity island 2', *Journal of Bacteriology*, 182(3), pp. 771–781. doi: 10.1128/JB.182.3.771-781.2000.

Lee, C. A. (2001) 'The HilA Box and Sequences outside It Determine the Magnitude of HilA-Dependent Activation of P', *Society*, 183(16), pp. 4876–4885. doi: 10.1128/JB.183.16.4876.

Lee, C. A., Jones, B. D. and Falkow, S. (1992) 'Identification of a *Salmonella* typhimurium invasion locus by selection for hyperinvasive mutants', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.89.5.1847.

Lee, V. T., Mazmanian, S. K. and Schneewind, O. (2001) 'A program of *Yersinia enterocolitica* type III secretion reactions is activated by specific signals', *Journal of Bacteriology*. doi: 10.1128/JB.183.17.4970-4978.2001.

Leirisalo-Repo, M. *et al.* (1997) 'Long term prognosis of reactive *Salmonella* arthritis', *Annals of the Rheumatic Diseases*, 56(9). doi: 10.1136/ard.56.9.516.

Li, X. *et al.* (2019) 'MUC1 is a receptor for the *Salmonella* SiiE adhesin that enables apical invasion into enterocytes', *PLoS Pathogens*, 15(2). doi: 10.1371/journal.ppat.1007566.

Lichtensteiger, C. A. and Vimr, E. R. (2003) 'Systemic and enteric colonization of pigs by a *hilA* signature-tagged mutant of *Salmonella* choleraesuis', *Microbial Pathogenesis*, 34(3), pp. 149–154. doi:

10.1016/S0882-4010(02)00196-1.

Lim, S. *et al.* (2006) 'Fis is required for proper regulation of *ssaG* expression in *Salmonella enterica* serovar Typhimurium', *Microbial Pathogenesis*. doi: 10.1016/j.micpath.2006.03.005.

Lim, S. *et al.* (2007) 'Mlc regulation of *Salmonella* pathogenicity island I gene expression via *hilE* repression', *Nucleic Acids Research*, 35(6). doi: 10.1093/nar/gkm060.

Lin, H. H. *et al.* (2020) 'Activation of apoptosis by *Salmonella* pathogenicity island-1 effectors through both intrinsic and extrinsic pathways in *Salmonella*-infected macrophages', *Journal of Microbiology, Immunology and Infection*. doi: 10.1016/j.jmii.2020.02.008.

Lin, S. L., Le, T. X. and Cowen, D. S. (2003) 'SptP, a *Salmonella* typhimurium type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation', *Cellular Microbiology*. doi: 10.1046/j.1462-5822.2003.t01-1-00274.x.

Lindén, S. K. *et al.* (2009) 'MUC1 limits *Helicobacter pylori* infection both by steric hindrance and by acting as a releasable decoy.', *PLoS pathogens*, 5(10). doi: 10.1371/journal.ppat.1000617.

Liss, V. *et al.* (2017) '*Salmonella enterica* Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition', *Cell Host & Microbe*, 0(0), pp. 390–402. doi: 10.1016/j.chom.2017.02.005.

Liu, B. *et al.* (2014) 'Structural diversity in *Salmonella* O antigens and its genetic basis', *FEMS Microbiology Reviews*. doi: 10.1111/1574-6976.12034.

Liu, Q. (2017) '*Salmonella* Choleraesuis outer membrane vesicles : Proteomics and immunogenicity', (March), pp. 1–10. doi: 10.1002/jobm.201700153.

Liu, W. *et al.* (2020) 'The Gli1-Snail axis contributes to *Salmonella* Typhimurium-induced disruption of intercellular junctions of intestinal epithelial cells', *Cellular Microbiology*. doi: 10.1111/cmi.13211.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method', *Methods*, 25(4). doi: 10.1006/meth.2001.1262.

Longo, A. *et al.* (2019) 'Characterizing *Salmonella enterica* Serovar Choleraesuis, var. Kunzendorf: A Comparative Case Study', *Frontiers in Veterinary Science*, 6(September), pp. 1–5. doi: 10.3389/fvets.2019.00316.

López-Garrido, J., Puerta-Fernández, E. and Casadesús, J. (2014) 'A eukaryotic-like 3′ untranslated region in *Salmonella enterica hilD* mRNA',

Nucleic Acids Research. doi: 10.1093/nar/gku222.

Lostroh, C. P., Bajaj, V. and Lee, C. A. (2000) 'The cis requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1', *Molecular Microbiology*, 37(2), pp. 300–315. doi: 10.1046/j.1365-2958.2000.01991.x.

Lou, L. *et al.* (2019) '*Salmonella* Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network', *Frontiers in Cellular and Infection Microbiology*. doi: 10.3389/fcimb.2019.00270.

Löwer, M. and Schneider, G. (2009) 'Prediction of type III secretion signals in genomes of gram-negative bacteria', *PLoS ONE*. doi: 10.1371/journal.pone.0005917.

Lucchini, S. *et al.* (2006) 'H-NS mediates the silencing of laterally acquired genes in bacteria', *PLoS Pathogens*, 2(8). doi: 10.1371/journal.ppat.0020081.

Lupolova, N. *et al.* (2017) 'Patchy promiscuity: machine learning applied to predict the host specificity of *Salmonella enterica* and *Escherichia coli*', *Microbial Genomics*, pp. 1–10. doi: 10.1099/mgen.0.000135.

Ma, S. *et al.* (2017) 'Prevalence, Antimicrobial Resistance, and Relatedness of *Salmonella* Isolated from Chickens and Pigs on Farms, Abattoirs, and Markets in Sichuan Province, China', *Foodborne Pathogens and Disease*, 14(11). doi: 10.1089/fpd.2016.2264.

Main-Hester, K. L. *et al.* (2008) 'Coordinate regulation of *Salmonella* pathogenicity island 1 (SPI1) and SPI4 in *Salmonella enterica* serovar typhimurium', *Infection and Immunity*, 76(3), pp. 1024–1035. doi: 10.1128/IAI.01224-07.

Majowicz, S. E. *et al.* (2010) 'The Global Burden of Nontyphoidal *Salmonella* Gastroenteritis', *Clinical Infectious Diseases*, 50(6), pp. 882–889. doi: 10.1086/650733.

Malik-Kale, P., Winfree, S. and Steele-Mortimer, O. (2012) 'The bimodal lifestyle of intracellular *Salmonella* in epithelial cells: Replication in the cytosol obscures defects in vacuolar replication', *PLoS ONE*, 7(6), pp. 1–10. doi: 10.1371/journal.pone.0038732.

Mallo, G. V. *et al.* (2008) 'SopB promotes phosphatidylinositol 3-phosphate formation on *Salmonella* vacuoles by recruiting Rab5 and Vps34', *Journal of Cell Biology*, 182(4), pp. 741–752. doi: 10.1083/jcb.200804131.

Mambu, J. *et al.* (2020) 'Rck of *Salmonella* Typhimurium Delays the Host Cell Cycle to Facilitate Bacterial Invasion', *Frontiers in Cellular and Infection Microbiology*. doi: 10.3389/fcimb.2020.586934.

Mandal, B. K. and Brennand, J. (1988) 'Bacteraemia in salmonellosis: A 15 year retrospective study from a regional infectious diseases unit', *British Medical Journal*, 297(6658). doi: 10.1136/bmj.297.6658.1242.

Mandlik, A. *et al.* (2011) 'RNA-seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression', *Cell Host and Microbe*. Elsevier Inc., 10(2), pp. 165–174. doi: 10.1016/j.chom.2011.07.007.

Mariani, V. *et al.* (2009) 'Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I', *Veterinary Immunology and Immunopathology*, 131(3–4). doi: 10.1016/j.vetimm.2009.04.006.

Marijke Keestra, A. *et al.* (2011) 'A *Salmonella* virulence factor activates the NOD1/NOD2 signaling pathway', *mBio*. doi: 10.1128/mBio.00266-11.

Marteyn, B. *et al.* (2010) 'Modulation of *Shigella* virulence in response to available oxygen in vivo', *Nature*. doi: 10.1038/nature08970.

Martinez-Sanguiné, A. Y. *et al.* (2020) ' *Salmonella* Dublin and Enteritidis comparative proteomics reveals differential expression of proteins involved in stress resistance, virulence and anaerobic metabolism ', *Infection and Immunity*. doi: 10.1128/iai.00606-20.

Martínez, L. C. *et al.* (2011) 'Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the *Salmonella* SPI-1 and SPI-2 virulence regulons through HilD', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2011.07674.x.

Mastroeni, P. *et al.* (2000) 'Antimicrobial actions of the nadph phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo', *Journal of Experimental Medicine*, 192(2). doi: 10.1084/jem.192.2.237.

McClelland, M. *et al.* (2004) 'Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid', *Nature Genetics*, 36(12), pp. 1268–1274. doi: 10.1038/ng1470.

McDermott, J. E. *et al.* (2011) 'Minireview: Computational prediction of type III and IV secreted effectors in gram-negative bacteria', *Infection and Immunity*, 79(1), pp. 23–32. doi: 10.1128/IAI.00537-10.

McDowell, M. A. *et al.* (2019) 'The *S.* Typhi effector StoD is an E3/E4 ubiquitin ligase which binds K48- And K63-linked diubiquitin', *Life Science Alliance*. doi: 10.26508/lsa.201800272.

McGhie, E. J., Hayward, R. D. and Koronakis, V. (2001) 'Cooperation between actin-binding proteins of invasive *Salmonella*: SipA potentiates SipC nucleation and bundling of actin', *EMBO Journal*, 20(9), pp. 2131–2139. doi: 10.1093/emboj/20.9.2131.

McLaughlin, L. M. *et al.* (2009) 'The *Salmonella* SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration', *PLoS Pathogens*, 5(11). doi: 10.1371/journal.ppat.1000671.

McQuiston, J. R. *et al.* (2004) 'Sequencing and Comparative Analysis of Flagellin Genes *fliC, fljB,* and *flpA* from *Salmonella'*, *Journal of Clinical Microbiology*, 42(5). doi: 10.1128/JCM.42.5.1923-1932.2004.

McQuiston, J. R. *et al.* (2008) 'Do *Salmonella* carry spare tyres?', *Trends in Microbiology*, 16(4). doi: 10.1016/j.tim.2008.01.009.

Mearin, F. *et al.* (2005) 'Dyspepsia and irritable bowel syndrome after a *Salmonella* gastroenteritis outbreak: One-year follow-up cohort study', *Gastroenterology*, 129(1). doi: 10.1053/j.gastro.2005.04.012.

MELZER, M. *et al.* (1965) '*SALMONELLA* INFECTIONS OF THE KIDNEY', *The Journal of urology*. doi: 10.1016/S0022-5347(17)63560-2.

Mercante, J. *et al.* (2009) 'Molecular Geometry of CsrA (RsmA) Binding to RNA and Its Implications for Regulated Expression', *Journal of Molecular Biology*. doi: 10.1016/j.jmb.2009.07.034.

Méresse, S. *et al.* (1999) 'The rab7 GTPase controls the maturation of *Salmonella* typhimurium-containing vacuoles in HeLa cells', *EMBO Journal*, 18(16). doi: 10.1093/emboj/18.16.4394.

Métris, A. *et al.* (2017) 'SalmoNet, an integrated network of ten *Salmonella* enterica strains reveals common and distinct pathways to host adaptation', *npj Systems Biology and Applications*. Springer US, 3(1), p. 31. doi: 10.1038/s41540-017-0034-z.

Meyerholz, D. K. and Stabel, T. J. (2003) 'Comparison of early ileal invasion by *Salmonella enterica* serovars choleraesuis and typhimurium', *Veterinary Pathology*, 40(4). doi: 10.1354/vp.40-4-371.

Miao, E. A. *et al.* (2003) '*Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton', *Molecular Microbiology*, 48(2), pp. 401–415. doi: 10.1046/j.1365-2958.2003.t01-1-03456.x.

Michiels, T. *et al.* (1990) 'Secretion of Yop proteins by yersiniae', *Infection and Immunity*. doi: 10.1128/iai.58.9.2840-2849.1990.

Miki, T. *et al.* (2004) 'Characterization of Salmonella pathogenicity island 1 type III secretion-dependent hemolytic activity in *Salmonella enterica* serovar Typhimurium', *Microbial Pathogenesis*, 37(2), pp. 65–72. doi: 10.1016/j.micpath.2004.04.006.

Miller, R. A. *et al.* (2018) 'The typhoid toxin produced by the Nontyphoidal

Salmonella enterica Serotype javiana is required for induction of a DNA damage response in Vitro and systemic spread in Vivo', *mBio*, 9(2). doi: 10.1128/mBio.00467-18.

Mills, D. M., Bajaj, V. and Lee, C. A. (1995) 'A 40 kb chromosomal fragment encoding *Salmonella* typhimurium invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome', *Molecular Microbiology*, 15(4), pp. 749–759. doi: 10.1111/j.1365-2958.1995.tb02382.x.

Le Minor, L. and Popoff, M. Y. (1987) 'Designation of *Salmonella enterica* sp. nov., nom. rev., as the Type and Only Species of the Genus *Salmonella*: Request for an Opinion', *International Journal of Systematic Bacteriology*, 37(4). doi: 10.1099/00207713-37-4-465.

Mirold, S. *et al.* (1999) 'Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella* typhimurium strain.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(17), pp. 9845–50. doi: 10.1073/pnas.96.17.9845.

Mizusaki, H. *et al.* (2008) 'Signal pathway in salt-activated expression of the Salmonella pathogenicity island 1 type III secretion system in *Salmonella enterica* serovar typhimurium', *Journal of Bacteriology*. doi: 10.1128/JB.01957-07.

Morgan, E. *et al.* (2004) 'Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium', *Molecular Microbiology*, 54(4), pp. 994–1010. doi: 10.1111/j.1365-2958.2004.04323.x.

Morgan, E. *et al.* (2007) 'SiiE is secreted by the *Salmonella enterica* serovar typhimurium pathogenicity island 4-encoded secretion system and contributes to intestinal colonization in cattle', *Infection and Immunity*, 75(3), pp. 1524–1533. doi: 10.1128/IAI.01438-06.

Morris, C. *et al.* (2003) '*Salmonella enterica* serovar Dublin strains which are Vi antigen-positive use type IVB pili for bacterial self-association and human intestinal cell entry', *Microbial Pathogenesis*, 35(6), pp. 279–284. doi: 10.1016/j.micpath.2003.08.001.

Mueller-Doblies, D., Speed, K. and Davies, R. H. (2013) 'A retrospective analysis of *Salmonella* serovars isolated from pigs in Great Britain between 1994 and 2010', *Preventive Veterinary Medicine*, 110(3–4). doi: 10.1016/j.prevetmed.2013.02.023.

Mukherjee, K. *et al.* (2000) 'Live *Salmonella* recruits N-ethylmaleimide-sensitive fusion protein on phagosomal membrane and promotes fusion with early endosome', *Journal of Cell Biology*, 148(4). doi: 10.1083/jcb.148.4.741.

'Multi-country outbreak of *Salmonella* Enteritidis sequence type (ST)11 infections linked to poultry products in the EU/EEA and the United Kingdom'

(2021) *EFSA Supporting Publications*, 18(3). doi: 10.2903/sp.efsa.2021.en-6486.

Murase, T. *et al.* (2000) 'Fecal excretion of *Salmonella enterica* serovar Typhimurium following a food-borne outbreak', *Journal of Clinical Microbiology*, 38(9). doi: 10.1128/jcm.38.9.3495-3497.2000.

Myeni, S. K. and Zhou, D. (2010) 'The C terminus of SipC binds and bundles F-actin to promote *Salmonella* invasion', *Journal of Biological Chemistry*, 285(18), pp. 13357–13363. doi: 10.1074/jbc.M109.094045.

Mylona, E. *et al.* (2020) ' Very long O-antigen chains of *Salmonella* Paratyphi A inhibit inflammasome activation and pyroptotic cell death ', *Cellular Microbiology*. doi: 10.1111/cmi.13306.

Narm, K. E., Kalafatis, M. and Slauch, J. M. (2020) 'HilD, HilC, and RtsA form homodimers and heterodimers to regulate expression of the *Salmonella* pathogenicity island i type iii secretion system', *Journal of Bacteriology*, 202(9). doi: 10.1128/JB.00012-20.

Navarre, W. W. *et al.* (2005) 'Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2005.04553.x.

Navarre, W. W. *et al.* (2006) 'Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella'*, *Science*. doi: 10.1126/science.1128794.

Neilson, K. A. *et al.* (2011) 'Less label, more free: Approaches in label-free quantitative mass spectrometry', *Proteomics*, 11(4), pp. 535–553. doi: 10.1002/pmic.201000553.

Ng, G. Z. *et al.* (2016) 'The MUC1 mucin protects against *Helicobacter pylori* pathogenesis in mice by regulation of the NLRP3 inflammasome', *Gut*, 65(7). doi: 10.1136/gutjnl-2014-307175.

Nichols, C. D. and Casanova, J. E. (2010) '*Salmonella*-directed recruitment of new membrane to invasion foci via the host exocyst complex', *Current Biology*, 20(14). doi: 10.1016/j.cub.2010.05.065.

Nickerson, C. A. and Achberger, E. C. (1995) 'Role of curved DNA in binding of Escherichia coli RNA polymerase to promoters', *Journal of Bacteriology*, 177(20). doi: 10.1128/jb.177.20.5756-5761.1995.

Niemann, G. S. *et al.* (2011) 'Discovery of novel secreted virulence factors from *Salmonella enterica* serovar typhimurium by proteomic analysis of culture supernatants', *Infection and Immunity*, 79(1), pp. 33–43. doi: 10.1128/IAI.00771-10.

Nisco, N. J. De (2018) ‘crossm The Biochemistry of Sensing : Enteric Pathogens Regulate Type III Secretion in Response to Environmental and Host Cues’, pp. 1–15.

Nomenclature Committee of the International Society for Microbiology (1934) ‘The genus *Salmonella* ligniéres, 1900: Issued by the *salmonella* subcommittee of the nomenclature committee of the international society for microbiology’, *Journal of Hygiene*, 34(3). doi: 10.1017/S0022172400034677.

Norris, F. A. *et al.* (1998) ‘SopB, a protein required for virulence of *Salmonella* dublin, is an inositol phosphate phosphatase.’, *Pnas*, 95(24), pp. 14057–14059. doi: 10.1073/pnas.95.24.14057.

Nuccio, S. and Bäumler, A. J. (2014) ‘Comparative Analysis of *Salmonella* Genomes Identifies a Metabolic’, 5(2), pp. 1–8. doi: 10.1128/mBio.00929-14.Editor.

Okoro, C. K. *et al.* (2015) ‘Signatures of Adaptation in Human Invasive *Salmonella* Typhimurium ST313 Populations from Sub-Saharan Africa’, *PLoS Neglected Tropical Diseases*, 9(3). doi: 10.1371/journal.pntd.0003611.

Olekhnovich, I. N. and Kadner, R. J. (2002) ‘DNA-binding activities of the HilC and HilD virulence regulatory proteins of *Salmonella enterica* serovar Typhimurium’, *Journal of Bacteriology*, 184(15), pp. 4148–4160. doi: 10.1128/JB.184.15.4148-4160.2002.

Olekhnovich, I. N. and Kadner, R. J. (2006) ‘Crucial roles of both flanking sequences in silencing of the hilA promoter in *Salmonella enterica*’, *Journal of Molecular Biology*, 357(2). doi: 10.1016/j.jmb.2006.01.007.

Olekhnovich, I. N. and Kadner, R. J. (2007) ‘Role of nucleoid-associated proteins Hha and H-NS in expression of *Salmonella enterica* activators HilD, HilC, and RtsA required for cell invasion’, *Journal of Bacteriology*. doi: 10.1128/JB.00905-07.

Olsen, J. E. *et al.* (2013) ‘The role of flagella and chemotaxis genes in host pathogen interaction of the host adapted *Salmonella enterica* serovar Dublin compared to the broad host range serovar S. Typhimurium’, *BMC Microbiology*, 13(1). doi: 10.1186/1471-2180-13-67.

Olsen, S. J. *et al.* (2003) ‘Outbreaks of typhoid fever in the United States, 1960-99’, *Epidemiology and Infection*, 130(1). doi: 10.1017/S0950268802007598.

Otto, A. *et al.* (2017) ‘An Easy and Fast Protocol for Affinity Bead-Based Protein Enrichment and Storage of Proteome Samples’, in *Methods in Enzymology*. doi: 10.1016/bs.mie.2016.09.012.

Panagi, I. *et al.* (2020) ‘*Salmonella* Effector SteE Converts the Mammalian

Serine/Threonine Kinase GSK3 into a Tyrosine Kinase to Direct Macrophage Polarization', *Cell Host and Microbe*. doi: 10.1016/j.chom.2019.11.002.

Panina, E. M. *et al.* (2005) 'A genome-wide screen identifies a *Bordetella* type III secretion effector and candidate effectors in other species', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2005.04823.x.

Pardon, P. *et al.* (1988) 'Ovine salmonellosis caused by *Salmonella* abortus ovis', *Annales de recherches vétérinaires. Annals of veterinary research*.

Park, D. *et al.* (2018) 'Visualization of the type III secretion mediated *salmonella*–host cell interface using cryo-electron tomography', *eLife*, 7. doi: 10.7554/eLife.39514.

Parra-Lopez, C. *et al.* (1994) 'A *Salmonella* protein that is required for resistance to antimicrobial peptides and transport of potassium', *EMBO Journal*, 13(17). doi: 10.1002/j.1460-2075.1994.tb06712.x.

Parry, C. M. *et al.* (2002) 'TYPHOID fever is a systemic infection with the bacterium', *The New England Journal of Medicine*, 347(22).

Parsons, B. N. *et al.* (2013) 'Invasive Non-Typhoidal *Salmonella* Typhimurium ST313 Are Not Host-Restricted and Have an Invasive Phenotype in Experimentally Infected Chickens', *PLoS Neglected Tropical Diseases*, 7(10), pp. 1–8. doi: 10.1371/journal.pntd.0002487.

Parsot, C. *et al.* (1995) 'Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.1995.tb02301.x.

Patel, J. C. and Galán, J. E. (2006) 'Differential activation and function of Rho GTPases during *Salmonella*-host cell interactions.', *The Journal of cell biology*, 175(3), pp. 453–63. doi: 10.1083/jcb.200605144.

Patel, S. and McCormick, B. A. (2014) 'Mucosal inflammatory response to *Salmonella* typhimurium infection', *Frontiers in Immunology*, 5(JUL), pp. 1–10. doi: 10.3389/fimmu.2014.00311.

Paulin, S. M. *et al.* (2002) 'Analysis of *Salmonella enterica* serotype-host specificity in calves: Avirulence of *S. enterica* serotype Gallinarum correlates with bacterial dissemination from mesenteric lymph nodes and persistence in vivo', *Infection and Immunity*, 70(12), pp. 6788–6797. doi: 10.1128/IAI.70.12.6788-6797.2002.

Paulin, S. M. *et al.* (2007) 'Net replication of *Salmonella enterica* serovars typhimurium and choleraesuis in porcine intestinal mucosa and nodes is associated with their differential virulence', *Infection and Immunity*, 75(8), pp. 3950–3960. doi: 10.1128/IAI.00366-07.

Pegues, D. A. *et al.* (1995) 'PhoP/PhoQ transcriptional repression of *Salmonella* typhimurium invasion genes: evidence for a role in protein secretion', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.1995.mmi_17010169.x.

Penner, J. L. (1988) 'International Committee on Systematic Bacteriology Taxonomic Subcommittee on Enterobacteriaceae: Minutes of the Meeting, 8 September 1986, Manchester, England', *International Journal of Systematic Bacteriology*, 38(2). doi: 10.1099/00207713-38-2-223.

Perrin, A. J. *et al.* (2004) 'Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system', *Current Biology*, 14(9). doi: 10.1016/j.cub.2004.04.033.

Pfaffl, M. W. (2001) 'A new mathematical model for relative quantification in real-time RT-PCR', *Nucleic Acids Research*. doi: 10.1093/nar/29.9.e45.

Pickard, D. *et al.* (2003) 'Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7', *Journal of Bacteriology*, 185(17), pp. 5055–5065. doi: 10.1128/JB.185.17.5055-5065.2003.

Poh, J. *et al.* (2008) 'SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling', *Cellular Microbiology*, 10(1), pp. 20–30. doi: 10.1111/j.1462-5822.2007.01010.x.

Porwollik, S. *et al.* (2004) 'Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays', *Journal of Bacteriology*, 186(17). doi: 10.1128/JB.186.17.5883-5898.2004.

Porwollik, S. *et al.* (2005) 'Differences in gene content between *Salmonella enterica* serovar enteritidis isolates and comparison to closely related serovars gallinarum and Dublin', *Journal of Bacteriology*. doi: 10.1128/JB.187.18.6545-6555.2005.

Potts, A. H. *et al.* (2019) 'Role of CsrA in stress responses and metabolism important for *Salmonella* virulence revealed by integrated transcriptomics', *PLoS ONE*. doi: 10.1371/journal.pone.0211430.

Powers, T. R. *et al.* (2021) 'Niche-specific profiling reveals transcriptional adaptations required for the cytosolic lifestyle of salmonella enterica', *bioRxiv*. doi: 10.1101/2021.01.11.426201.

Prashar, A. *et al.* (2017) 'Rab GTPases in Immunity and Inflammation', *Frontiers in Cellular and Infection Microbiology*, 7(September), p. 435. doi: 10.3389/fcimb.2017.00435.

Pulford, C. V. *et al.* (2021) 'Stepwise evolution of *Salmonella* Typhimurium ST313 causing bloodstream infection in Africa', *Nature Microbiology*, 6(3).

doi: 10.1038/s41564-020-00836-1.

Pullinger, G. D. *et al.* (2007) 'Systemic translocation of *Salmonella enterica* serovar Dublin in cattle occurs predominantly via efferent lymphatics in a cell-free niche and requires type III secretion system 1 (T3SS-1) but not T3SS-2', *Infection and Immunity*, 75(11), pp. 5191–5199. doi: 10.1128/IAI.00784-07.

Van Putten, J. P. M. and Strijbis, K. (2017) 'Transmembrane Mucins: Signaling Receptors at the Intersection of Inflammation and Cancer', *Journal of Innate Immunity*. doi: 10.1159/000453594.

El qaidi, S. *et al.* (2017) 'NleB/SseK effectors from *Citrobacter rodentium, Escherichia coli,* and *Salmonella enterica* display distinct differences in host substrate specificity', *Journal of Biological Chemistry*, 292(3), pp. 11423–11430. doi: 10.1074/jbc.M117.790675.

Radtke, A. L. *et al.* (2010) 'Analysis of interactions of salmonella type three secretion mutants with 3-D intestinal epithelial cells', *PLoS ONE*, 5(12). doi: 10.1371/journal.pone.0015750.

Raffatellu, M., Wilson, R. P., *et al.* (2005) 'SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella enterica* serotype typhimurium invasion of epithelial cells', *Infection and Immunity*. doi: 10.1128/IAI.73.1.146-154.2005.

Raffatellu, M., Chessa, D., *et al.* (2005) 'The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa', *Infection and Immunity*. doi: 10.1128/IAI.73.6.3367-3374.2005.

Rakov, A. V. *et al.* (2019) 'Association of *Salmonella* virulence factor alleles with intestinal and invasive serovars', *BMC Genomics*. doi: 10.1186/s12864-019-5809-8.

Rankin, J. D. and Taylor, R. J. (1966) 'The estimation of doses of *Salmonella* typhimurium suitable for the experimental production of disease in calves', *The Veterinary Record*, 78(21), pp. 706–707.

Rathman, M., Barker, L. P. and Falkow, S. (1997) 'The unique trafficking pattern of *Salmonella* typhimurium-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry', *Infection and Immunity*, 65(4), pp. 1475–1485.

Rauch, I. *et al.* (2017) 'NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8', *Immunity*. Elsevier Inc., 46(4), pp. 649–659. doi: 10.1016/j.immuni.2017.03.016.

Reddy, E. A., Shaw, A. V. and Crump, J. A. (2010) 'Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis', *The*

Lancet Infectious Diseases. doi: 10.1016/S1473-3099(10)70072-4.

Reyes Ruiz, V. M. *et al.* (2017) 'Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome', *Proceedings of the National Academy of Sciences*, 114(50), p. 201710433. doi: 10.1073/pnas.1710433114.

Rice, C. J. *et al.* (2015) 'Transcriptional and post-transcriptional modulation of SPI1 and SPI2 expression by ppGpp, RpoS and DksA in *Salmonella enterica* sv Typhimurium', *PLoS ONE*, 10(6), pp. 1–18. doi: 10.1371/journal.pone.0127523.

Richardson, E. J. *et al.* (2011) 'Genome sequences of *salmonella enterica* serovar typhimurium, choleraesuis, dublin, and gallinarum strains of well-defined virulence in food-producing animals', *Journal of Bacteriology*, 193(12), pp. 3162–3163. doi: 10.1128/JB.00394-11.

Richter-Dahlfors, a, Buchan, a M. and Finlay, B. B. (1997) 'Murine salmonellosis studied by confocal microscopy: *Salmonella* typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo.', *The Journal of experimental medicine*, 186(4), pp. 569–580. doi: 10.1084/jem.186.4.569.

Roche, S. M. *et al.* (2018) '*Salmonella* Typhimurium Invalidated for the Three Currently Known Invasion Factors Keeps Its Ability to Invade Several Cell Models', *Frontiers in Cellular and Infection Microbiology*, 8(August). doi: 10.3389/fcimb.2018.00273.

Rodríguez, D. M. and Suárez, M. C. (2014) '*Salmonella* spp. in the pork supply chain: A risk approach', *Revista Colombiana de Ciencias Pecuarias*.

Roe, A. J. *et al.* (2003) 'Heterogeneous surface expression of EspA translocon filaments by *Escherichia coli* O157:H7 is controlled at the posttranscriptional level', *Infection and Immunity*. doi: 10.1128/IAI.71.10.5900-5909.2003.

Rolain, J. M., Colson, P. and Raoult, D. (2007) 'Recycling of chloroquine and its hydroxyl analogue to face bacterial, fungal and viral infections in the 21st century', *International Journal of Antimicrobial Agents*. doi: 10.1016/j.ijantimicag.2007.05.015.

Roof, M. B. and Doitchinoff, D. D. (1995) 'Safety, efficacy, and duration of immunity induced in swine by use of an avirulent live *Salmonella* choleraesuis-containing vaccine', *American Journal of Veterinary Research*, 56(1).

Rosqvist, R. *et al.* (1995) 'Functional conservation of the secretion and translocation machinery for virulence proteins of yersiniae, salmonellae and shigellae.', *The EMBO Journal*. doi: 10.1002/j.1460-2075.1995.tb00092.x.

Rosselin, M. *et al.* (2010) 'Rck of *Salmonella enterica*, subspecies enterica serovar Enteritidis, mediates Zipper-like internalization', *Cell Research*, 20(6). doi: 10.1038/cr.2010.45.

Rothhardt, J. E. *et al.* (2014) 'The orphan regulator ReiD of *Salmonella enterica* is essential for myo-inositol utilization', *Molecular Microbiology*. doi: 10.1111/mmi.12788.

Ryan, M. P., O'Dwyer, J. and Adley, C. C. (2017) 'Evaluation of the Complex Nomenclature of the Clinically and Veterinary Significant Pathogen *Salmonella'*, *BioMed Research International*. doi: 10.1155/2017/3782182.

Rychlik, I. and Barrow, P. A. (2005) '*Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection', *FEMS Microbiology Reviews*, 29(5), pp. 1021–1040. doi: 10.1016/j.femsre.2005.03.005.

Rydström, A. and Wick, M. J. (2007) ' Monocyte Recruitment, Activation, and Function in the Gut-Associated Lymphoid Tissue during Oral *Salmonella* Infection ', *The Journal of Immunology*. doi: 10.4049/jimmunol.178.9.5789.

Saha, P. *et al.* (2019) 'The bacterial siderophore enterobactin confers survival advantage to *Salmonella* in macrophages', *Gut Microbes*, 10(3). doi: 10.1080/19490976.2018.1546519.

Saini, S. *et al.* (2010) 'The role of coupled positive feedback in the expression of the SPI1 type three secretion system in *Salmonella'*, *PLoS Pathogens*, 6(7), pp. 1–16. doi: 10.1371/journal.ppat.1001025.

Saini, S., Pearl, J. A. and Rao, C. V. (2009) 'Role of FimW, FimY, and FimZ in regulating the expression of type I fimbriae in *salmonella enterica* serovar typhimurium', *Journal of Bacteriology*, 191(9). doi: 10.1128/JB.01694-08.

Sakharkar, K. R. and Chow, V. T. K. (2005) 'Strategies for genome reduction in microbial genomes.', *Genome informatics. International Conference on Genome Informatics*. doi: 10.11234/gi1990.16.2_69.

Salcedo, S. P. and Holden, D. W. (2003) 'SseG, a virulence protein that targets *Salmonella* to the Golgi network', *EMBO Journal*. doi: 10.1093/emboj/cdg517.

Saleh, S. *et al.* (2019) '*Salmonella* Typhi, paratyphi A, enteritidis and typhimurium core proteomes reveal differentially expressed proteins linked to the cell surface and pathogenicity', *PLoS Neglected Tropical Diseases*. doi: 10.1371/journal.pntd.0007416.

Salmond, G. P. C. and Reeves, P. J. (1993) 'Membrance traffic wardens and protein secretion in Gram-negative bacteria', *Trends in Biochemical Sciences*. doi: 10.1016/0968-0004(93)90080-7.

Samudrala, R., Heffron, F. and McDermott, J. E. (2009) 'Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type iii secretion systems', *PLoS Pathogens*, 5(4). doi: 10.1371/journal.ppat.1000375.

Sanchis, R., Abadie, G. and Pardon, P. (1995) '*Salmonella* Abortusovis experimental infection induced by the conjunctival route: clinical, serological and bacteriological study of the dose effect in female lambs', *Veterinary Research*, 26(2). doi: 10.1016/0928-4249(96)82417-5.

Sansone, A. *et al.* (2002) 'The role of two periplasmic copper- and zinc-cofactored superoxide dismutases in the virulence of *Salmonella* choleraesuis', *Microbiology*, 148(3), pp. 719–726.

Schechter, L. M. and Lee, C. A. (2001) 'AraC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella* typhimurium *hilA* promoter', *Molecular Microbiology*, 40(6), pp. 1289–1299. doi: 10.1046/j.1365-2958.2001.02462.x.

Scherer, C. A., Cooper, E. and Miller, S. I. (2000) 'The *Salmonella* type III secretion translocon protein SspC is inserted into the epithelial cell plasma membrane upon infection', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2000.02066.x.

Schierack, P. *et al.* (2006) 'Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine', *Histochemistry and Cell Biology*, 125(3), pp. 293–305. doi: 10.1007/s00418-005-0067-z.

Schmitt, C. K. *et al.* (2001) 'Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis', *Infection and Immunity*, 69(9), pp. 5619–5625. doi: 10.1128/IAI.69.9.5619-5625.2001.

Schroeder, N. *et al.* (2010) 'The virulence protein SopD2 regulates membrane dynamics of Salmonella-containing vacuoles', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1001002.

Sévellec, Y. *et al.* (2019) 'Genetic diversity of *salmonella* derby from the poultry sector in europe', *Pathogens*, 8(2). doi: 10.3390/pathogens8020046.

Shea, J. E. *et al.* (1996) 'Identification of a virulence locus encoding a second type III secretion system in *Salmonella* typhimurium.', *Proceedings of the National Academy of Sciences of the United States of America*, 93(6), pp. 2593–2597. doi: 10.1073/pnas.93.6.2593.

Shea, J. E. *et al.* (1999) 'Influence of the *Salmonella* typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the

mouse', *Infection and Immunity*. doi: 10.1128/iai.67.1.213-219.1999.

Sherry, A. E. *et al.* (2011) 'Characterisation of proteins extracted from the surface of *Salmonella* Typhimurium grown under SPI-2-inducing conditions by LC-ESI/MS/MS sequencing', *Proteomics*, 11(3), pp. 361–370. doi: 10.1002/pmic.200900802.

Shi, L. *et al.* (2009) 'Proteome of *Salmonella Enterica* Serotype Typhimurium Grown in a Low Mg2+/pH Medium', *Proteomics*, 38105(509), pp. 388–397. doi: 10.4172/jpb.1000099.Proteome.

Sievers, F. *et al.* (2014) 'Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega', *Molecular Systems Biology*, 7(1), pp. 539–539. doi: 10.1038/msb.2011.75.

Da Silva, C. *et al.* (2017) 'The Peyer's patch mononuclear phagocyte system at steady state and during infection', *Frontiers in Immunology*. doi: 10.3389/fimmu.2017.01254.

Singer, H. M. *et al.* (2012) 'Selective purification of recombinant neuroactive peptides using the flagellar type III secretion system', *mBio*. doi: 10.1128/mBio.00115-12.

Sittka, A. *et al.* (2008) 'Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq', *PLoS Genetics*. doi: 10.1371/journal.pgen.1000163.

Skjolaas, K. A. *et al.* (2006) 'Effects of *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) on chemokine and cytokine expression in swine ileum and jejunal epithelial cells', *Veterinary Immunology and Immunopathology*, 111(3–4), pp. 199–209. doi: 10.1016/j.vetimm.2006.01.002.

Skjolaas, K. A. *et al.* (2007) 'Effects of *Salmonella enterica* serovar Typhimurium, or serovar Choleraesuis, *Lactobacillus reuteri* and *Bacillus licheniformis* on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPEC-J2', *Veterinary Immunology and Immunopathology*. doi: 10.1016/j.vetimm.2006.10.012.

Smith, H. W. and Jones, J. E. (1967) 'Observations on experimental oral infection with *Salmonella* dublin in calves and *Salmonella* choleraesuis in pigs.', *The Journal of pathology and bacteriology*. doi: 10.1002/path.1700930114.

Smith, T. (1884) 'The hog-cholera group of bacteria', *US Department of Agriculture Bureau of Animal Industry Bulletin*, 6(6), p. 40.

Smith, T. (1900) 'Variation among pathogenic bacteria', *Journal of the Boston Society of Medical Sciences*, 4(5), pp. 95–109.

Solano-Collado, V. *et al.* (2021) 'A Small-Scale shRNA Screen in Primary Mouse Macrophages Identifies a Role for the Rab GTPase Rab1b in Controlling *Salmonella* Typhi Growth', *Frontiers in Cellular and Infection Microbiology*, 11. doi: 10.3389/fcimb.2021.660689.

Song, X. *et al.* (2019) 'Transcriptome analysis of virulence gene regulation by the ATP-dependent Lon protease in *Salmonella* Typhimurium', *Future Microbiology*, 14(13). doi: 10.2217/fmb-2019-0118.

Sory, M. -P and Cornelis, G. R. (1994) 'Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.1994.tb02191.x.

Spano, S. *et al.* (2016) 'A Bacterial Pathogen Targets a Host Rab-Family GTPase Defense Pathway with a GAP', *Cell Host Microbe*, 19(2), pp. 216–226. doi: 10.1016/j.chom.2016.01.004.

Spanò, S. and Galán, J. E. (2012) 'A Rab32-dependent pathway contributes to *Salmonella* typhi host restriction.', *Science (New York, N.Y.)*, 338(6109), pp. 960–3. doi: 10.1126/science.1229224.

Spanò, S., Liu, X. and Galán, J. E. (2011) 'Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host *Salmonella*.', *Proceedings of the National Academy of Sciences of the United States of America*, 108(45), pp. 18418–23. doi: 10.1073/pnas.1111959108.

Sprinz, H. *et al.* (1966) 'Histopathology of the upper small intestines in typhoid fever - Biopsy study of experimental disease in man', *The American Journal of Digestive Diseases*, 11(8). doi: 10.1007/BF02233509.

Srikumar, S. *et al.* (2015) 'RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of *Salmonella* Typhimurium', *PLoS Pathogens*, 11(11), pp. 1–26. doi: 10.1371/journal.ppat.1005262.

Stebbins, C. E. and Galán, J. E. (2001) 'Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion', *Nature*. doi: 10.1038/35102073.

Stecher, B. *et al.* (2007) '*Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota', *PLoS Biology*, 5(10). doi: 10.1371/journal.pbio.0050244.

Steele-Mortimer, O. *et al.* (1999) 'Biogenesis of *Salmonella* typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway', *Cellular Microbiology*, 1(1). doi: 10.1046/j.1462-5822.1999.00003.x.

Stein, M. A. *et al.* (1996) 'Identification of a *Salmonella* virulence gene

required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells', *Molecular Microbiology*, 20(1), pp. 151–164. doi: 10.1111/j.1365-2958.1996.tb02497.x.

Steinberg, T. H. (1994) 'Cellular transport of drugs', *Clinical Infectious Diseases*, 19(5). doi: 10.1093/clinids/19.5.916.

Steinmann, R. and Dersch, P. (2013) 'Thermosensing to adjust bacterial virulence in a fluctuating environment', *Future Microbiology*. doi: 10.2217/fmb.12.129.

Storek, K. M. and Monack, D. M. (2015) 'Bacterial recognition pathways that lead to inflammasome activation', *Immunological Reviews*. doi: 10.1111/imr.12289.

Sturm, A. *et al.* (2011) 'The cost of virulence: Retarded growth of *salmonella* typhimurium cells expressing type iii secretion system 1', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1002143.

Suijkerbuijk, A. W. M. *et al.* (2017) 'The economic burden of a *Salmonella* Thompson outbreak caused by smoked salmon in the Netherlands, 2012-2013', *European Journal of Public Health*, 27(2). doi: 10.1093/eurpub/ckw205.

Sun, H. *et al.* (2016) 'A Family of Salmonella Type III Secretion Effector Proteins Selectively Targets the NF-κB Signaling Pathway to Preserve Host Homeostasis', *PLoS Pathogens*, 12(3), pp. 1–19. doi: 10.1371/journal.ppat.1005484.

Swords, W. E., Cannon, B. M. and Benjamin, W. H. (1997) 'Avirulence of LT2 strains of *Salmonella* typhimurium results from a defective rpoS gene', *Infection and Immunity*, 65(6), pp. 2451–2453.

Szeto, J. *et al.* (2009) '*Salmonella*-containing vacuoles display centrifugal movement associated with cell-to-cell transfer in epithelial cells', *Infection and Immunity*, 77(3), pp. 996–1007. doi: 10.1128/IAI.01275-08.

Takaya, A. *et al.* (2002) 'The ATP-dependent lon protease of *Salmonella* enterica serovar Typhimurium regulates invasion and expression of genes carried on *Salmonella* pathogenicity island 1', *Journal of Bacteriology*. doi: 10.1128/JB.184.1.224-232.2002.

Takaya, A. *et al.* (2005) 'Degradation of the HilC and HilD regulator proteins by ATP-dependent Lon protease leads to downregulation of *Salmonella* pathogenicity island 1 gene expression', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2004.04425.x.

Tam, M. A., Sundquist, M. and Wick, M. J. (2008) 'Early cellular responses to *Salmonella* infection: dendritic cells, monocytes, and more', *Immunological*

Reviews, pp. 140–162.

Tambassi, M. *et al.* (2020) 'Mutation of *hilD* in a *Salmonella* Derby lineage linked to swine adaptation and reduced risk to human health', *Scientific Reports*. Nature Publishing Group UK, 10(1), pp. 1–13. doi: 10.1038/s41598-020-78443-7.

Thiennimitr, P. *et al.* (2011) 'Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota', *Proceedings of the National Academy of Sciences*, 108(42), pp. 17480–17485. doi: 10.1073/pnas.1107857108.

Thiennimitr, Parameth *et al.* (2011) 'Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota', *Proceedings of the National Academy of Sciences of the United States of America*, 108(42), pp. 17480–17485. doi: 10.1073/pnas.1107857108.

Thomas, P. *et al.* (2015) 'Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples', *Biotechnology Reports*, 8. doi: 10.1016/j.btre.2015.08.003.

Thompson Bastin, M. L. *et al.* (2016) 'An unusual case of *Salmonella* Enteritidis causing pneumonia, septic shock and multiple organ failure in an immunocompetent patient', *IDCases*, 6. doi: 10.1016/j.idcr.2016.10.004.

Threlfall, E. J. (2000) 'Epidemic *Salmonella* typhimurium DT 104 - A truly international multiresistant clone', *Journal of Antimicrobial Chemotherapy*, 46(1). doi: 10.1093/jac/46.1.7.

Tiwari, R. P. *et al.* (2004) 'Adaptive acid tolerance response in *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Typhi', *Journal of Basic Microbiology*, 44(2). doi: 10.1002/jobm.200310333.

Torpdahl, M. and Ahrens, P. (2004) 'Population structure of *Salmonella* investigated by amplified fragment length polymorphism', *Journal of Applied Microbiology*, 97(3). doi: 10.1111/j.1365-2672.2004.02337.x.

Trombert, A. N., Rodas, P. I. and Mora, G. C. (2011) 'Reduced invasion to human epithelial cell lines of *Salmonella enterica* serovar Typhi carrying *S*. Typhimurium sopD2', *FEMS Microbiology Letters*. doi: 10.1111/j.1574-6968.2011.02347.x.

Trongjit, S. *et al.* (2017) 'Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces', *Microbiology and Immunology*, 61(1). doi: 10.1111/1348-0421.12462.

Tsolis, R. M. *et al.* (1999) 'Identification of a Putative *Salmonella enterica*

Serotype Typhimurium Host Range Factor with Homology to IpaH and YopM by Signature-Tagged Mutagenesis Identification of a Putative *Salmonella enterica* Serotype Typhimurium Host Range Factor with Homology to', *Infection and immunity*, 67(12), pp. 6385–6393.

Tsui, I. S. M. *et al.* (2003) 'The type IVB pili of *Salmonella enterica* serovar Typhi bind to the cystic fibrosis transmembrane conductance regulator', *Infection and Immunity*, 71(10). doi: 10.1128/IAI.71.10.6049-6050.2003.

Tucker, S. C. and Galán, J. E. (2000) 'Complex function for SicA, a *Salmonella enterica* serovar typhimurium type III secretion-associated chaperone', *Journal of Bacteriology*. doi: 10.1128/JB.182.8.2262-2268.2000.

Uchiya, K. I. *et al.* (1999) 'A *Salmonella* virulence protein that inhibits cellular trafficking', *EMBO Journal*, 18(14). doi: 10.1093/emboj/18.14.3924.

Uthe, J. J. *et al.* (2007) 'Porcine differential gene expression in response to *Salmonella enterica* serovars Choleraesuis and Typhimurium', *Molecular Immunology*, 44(11). doi: 10.1016/j.molimm.2007.01.016.

Uzzau, S *et al.* (2000) 'Host adapted serotypes of *Salmonella enterica*.', *Epidemiology and infection*, 125(2), pp. 229–255. doi: 10.1017/S0950268899004379.

Uzzau, S. *et al.* (2001) '*Salmonella enterica* serovar-host specificity does not correlate with the magnitude of intestinal invasion in sheep', *Infection and Immunity*, 69(5), pp. 3092–3099. doi: 10.1128/IAI.69.5.3092-3099.2001.

Uzzau, S., Hovi, M. and Stocker, B. A. D. (1999) 'Application of ribotyping and IS200 fingerprinting to distinguish the five *Salmonella* serotype O6,7:c:1,5 groups: Choleraesuis sensu stricto, choleraesuis var. Kunzendorf, Choleraesuis var. Decatur, Paratyphi C, and Typhisuis', *Epidemiology and Infection*, 123(1). doi: 10.1017/S0950268899002654.

Vakulskas, C. A. *et al.* (2015) 'Regulation of Bacterial Virulence by Csr (Rsm) Systems', *Microbiology and Molecular Biology Reviews*. doi: 10.1128/mmbr.00052-14.

Valdivia, R. H. and Falkow, S. (1997) 'Fluorescence-based isolation of bacterial genes expressed within host cells', *Science*. doi: 10.1126/science.277.5334.2007.

Valenzuela, L. M. *et al.* (2015) 'Pseudogenization of *sopA* and *sopE2* is functionally linked and contributes to virulence of *Salmonella enterica* serovar Typhi', *Infection, Genetics and Evolution*. doi: 10.1016/j.meegid.2015.04.021.

Veenendaal, A. K. J. *et al.* (2007) 'The type III secretion system needle tip complex mediates host cell sensing and translocon insertion', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2007.05620.x.

Veldhuizen, E. J. A. *et al.* (2009) '*Salmonella* serovar specific upregulation of porcine defensins 1 and 2 in a jejunal epithelial cell line', *Veterinary Microbiology*. doi: 10.1016/j.vetmic.2008.09.072.

Vernikos, G. S. and Parkhill, J. (2006) 'Interpolated variable order motifs for identification of horizontally acquired DNA: Revisiting the Salmonella pathogenicity islands', *Bioinformatics*. doi: 10.1093/bioinformatics/btl369.

Vimal, D. B. *et al.* (2000) 'Intestinal mucins: The binding sites for *Salmonella* typhimurium', *Molecular and Cellular Biochemistry*, 204(1–2). doi: 10.1023/a:1007015312036.

Viola, M. F. and Boeckxstaens, G. (2020) 'Intestinal resident macrophages: Multitaskers of the gut', *Neurogastroenterology and Motility*. doi: 10.1111/nmo.13843.

Vohra, P., Vrettou, C., *et al.* (2019) 'Nature and consequences of interactions between *Salmonella enterica* serovar Dublin and host cells in cattle', *Veterinary Research*. doi: 10.1186/s13567-019-0720-5.

Vohra, P., Chaudhuri, R. R., *et al.* (2019) 'Retrospective application of transposon-directed insertion-site sequencing to investigate niche-specific virulence of *Salmonella* Typhimurium in cattle', *BMC Genomics*. BMC Genomics, 20(1), pp. 1–12. doi: 10.1186/s12864-018-5319-0.

Volk, M. *et al.* (2019) 'Transcriptional and Post-transcriptional Regulatory Mechanisms Controlling Type III Secretion'. doi: 10.1007/82_2019_168.

Wachter, C. *et al.* (1999) 'Insertion of EspD into epithelial target cell membranes by infecting enteropathogenic *Escherichia coli*', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.1999.01303.x.

Wales, A. D. and Davies, R. H. (2017) '*Salmonella* Vaccination in Pigs: A Review', *Zoonoses and Public Health*. doi: 10.1111/zph.12256.

Wall, D. M. *et al.* (2007) 'Identification of the *Salmonella enterica* serotype Typhimurium SipA domain responsible for inducing neutrophil recruitment across the intestinal epithelium', *Cellular Microbiology*, 9(9), pp. 2299–2313. doi: 10.1111/j.1462-5822.2007.00960.x.

Wallis, T. S. and Galyov, E. E. (2000) 'Molecular basis of *Salmonella* - induced enteritis', *Molecualar Microbiology*, 36(5), pp. 997–1005.

Walthers, D. *et al.* (2007) 'The response regulator SsrB activates expression of diverse *Salmonella* pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2007.05800.x.

Wang, J. Y. *et al.* (2006) 'Bacteraemia due to ciprofloxacin-resistant

Salmonella enterica serotype Choleraesuis in adult patients at a university hospital in Taiwan, 1996-2004', *Epidemiology and Infection*, 134(5). doi: 10.1017/S095026880600608X.

Wang, Y. *et al.* (2011) 'High-accuracy prediction of bacterial type III secreted effectors based on position-specific amino acid composition profiles', *Bioinformatics*. doi: 10.1093/bioinformatics/btr021.

Waterman, S. R. and Small, P. L. C. (1998) 'Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources', *Applied and Environmental Microbiology*, 64(10). doi: 10.1128/aem.64.10.3882-3886.1998.

Watson, P. R. *et al.* (1998) 'Mutation of invH , but Not stn , Reduces *Salmonella* -Induced Enteritis in Cattle Mutation of invH , but Not stn , Reduces *Salmonella* -Induced Enteritis in Cattle', 66(4), pp. 1432–1438.

Watson, P. R. *et al.* (2000) 'Interaction of *Salmonella* serotypes with porcine macrophages in vitro does not correlate with virulence', *Microbiology*, 146(7), pp. 1639–1649.

Westermann, A. J. *et al.* (2019) 'The major RNA-binding protein ProQ impacts virulence gene expression in *Salmonella enterica* serovar typhimurium', *mBio*. doi: 10.1128/mBio.02504-18.

Wheeler, N. E., Gardner, P. P. and Barquist, L. (2018) 'Machine learning identifies signatures of host adaptation in the bacterial pathogen *Salmonella enterica*', *PLoS Genetics*. doi: 10.1371/journal.pgen.1007333.

Whitlock, G. C. *et al.* (2008) 'Construction of a reporter system to study *Burkholderia mallei* type III secretion and identification of the BopA effector protein function in intracellular survival', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(SUPPL. 1). doi: 10.1016/S0035-9203(08)70029-4.

Wilmes-Riesenberg, M. R. *et al.* (1996) 'Role of the acid tolerance response in virulence of *Salmonella* typhimurium', *Infection and Immunity*, 64(4). doi: 10.1128/iai.64.4.1085-1092.1996.

Winter, S. E. *et al.* (2009) 'Contribution of flagellin pattern recognition to intestinal inflammation during *Salmonella enterica* serotype Typhimurium infection', *Infection and Immunity*. doi: 10.1128/IAI.01341-08.

Winter, S. E. *et al.* (2010) 'Gut inflammation provides a respiratory electron acceptor for *Salmonella'*, *Nature*, 467(7314). doi: 10.1038/nature09415.

Wolfson, E. B. *et al.* (2020) 'The interaction of *Escherichia coli* O157:H7 and *Salmonella* typhimurium flagella with host cell membranes and cytoskeletal

components', *Microbiology (United Kingdom)*, 166(10). doi: 10.1099/mic.0.000959.

Wood, M. W. *et al.* (1996) 'SopE, a secreted protein of *Salmonella* dublin, is translocated into the target eukaryotic cell via a sip-dependent mechanism and promotes bacterial entry', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.1996.00116.x.

Wood, M. W. *et al.* (1998) 'Identification of a pathogenicity island required for *Salmonella* enteropathogenicity', *Molecular Microbiology*, 29(3), pp. 883–891. doi: 10.1046/j.1365-2958.1998.00984.x.

Wood, M. W. *et al.* (2000a) 'The secreted effector protein of *Salmonella* dublin, SopA, is translocated into eukaryotic cells and influences the induction of enteritis', *Cellular Microbiology*, 2(4), pp. 293–303. doi: 10.1046/j.1462-5822.2000.00054.x.

Wood, M. W. *et al.* (2000b) 'The secreted effector protein of *Salmonella* dublin, SopA, is translocated into eukaryotic cells and influences the induction of enteritis', *Cellular Microbiology*, 2(4), pp. 293–303. doi: 10.1046/j.1462-5822.2000.00054.x.

Worley, M. J., Ching, K. H. L. and Heffron, F. (2000) '*Salmonella* SsrB activates a global regulon of horizontally acquired genes', *Molecular Microbiology*, 36(3), pp. 749–761. doi: 10.1046/j.1365-2958.2000.01902.x.

Xu, Y. *et al.* (2019) 'A Bacterial Effector Reveals the V-ATPase-ATG16L1 Axis that Initiates Xenophagy', *Cell*. doi: 10.1016/j.cell.2019.06.007.

Yang, Y. *et al.* (2010) 'Computational prediction of type III secreted proteins from gram-negative bacteria', *BMC Bioinformatics*. doi: 10.1186/1471-2105-11-S1-S47.

Yang, Y. A. *et al.* (2018) 'In vivo tropism of *Salmonella* Typhi toxin to cells expressing a multiantennal glycan receptor', *Nature Microbiology*, 3(2). doi: 10.1038/s41564-017-0076-4.

Ye, J. *et al.* (2012) 'Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction.', *BMC bioinformatics*, 13. doi: 10.1186/1471-2105-13-134.

Yeh, H. Y., Kojima, K. and Mobley, J. A. (2018) 'Epitope mapping of *Salmonella* flagellar hook-associated protein, FlgK, with mass spectrometry-based immuno-capture proteomics using chicken (Gallus gallus domesticus) sera', *Veterinary Immunology and Immunopathology*. doi: 10.1016/j.vetimm.2018.05.006.

Young, A. M. and Palmer, A. E. (2017) 'Methods to Illuminate the Role of *Salmonella* Effector Proteins during Infection: A Review', *Frontiers in Cellular*

and Infection Microbiology, 7(August). doi: 10.3389/fcimb.2017.00363.

Yu, H. B. *et al.* (2014) 'Autophagy facilitates *Salmonella* replication in HeLa cells', *mBio*, 5(2). doi: 10.1128/mBio.00865-14.

Yu, X. J. *et al.* (2010) 'pH Sensing by Intracellular *Salmonella* Induces Effector Translocation', *Science*, 328(5981), pp. 1040–1043. doi: 10.1126/science.1189000.

Yu, X. J., Liu, M. and Holden, D. W. (2004) 'SsaM and SpiC interact and regulate secretion of *Salmonella* Pathogenicity Island 2 type III secretion system effectors and translocators', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2004.04297.x.

Yu, Y. *et al.* (2003) 'TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism', *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 285(2 48-2). doi: 10.1152/ajpgi.00503.2002.

Yue, M. *et al.* (2012) 'Diversification of the *Salmonella* Fimbriae: A model of macro- and microevolution', *PLoS ONE*, 7(6). doi: 10.1371/journal.pone.0038596.

Yue, M. *et al.* (2015) 'Allelic variation contributes to bacterial host specificity', *Nature Communications*. doi: 10.1038/ncomms9754.

Zhang, K. *et al.* (2018) 'Minimal SPI1-T3SS effector requirement for *Salmonella* enterocyte invasion and intracellular proliferation in vivo', *PLoS Pathogens*, 14(3). doi: 10.1371/journal.ppat.1006925.

Zhang, S. *et al.* (2002) 'Phage mediated horizontal transfer of the sopE1 gene increases enteropathogenicity of *Salmonella enterica* serotype Typhimurium for calves', *FEMS Microbiology Letters*, 217(2). doi: 10.1016/S0378-1097(02)01094-7.

Zhang, Y. *et al.* (2015) 'Bacterial protein AvrA stabilizes intestinal epithelial tight junctions via blockage of the C-Jun N-terminal kinase pathway.', *Tissue barriers*, 3(1–2), p. e972849. doi: 10.4161/21688362.2014.972849.

Zhao, W. *et al.* (2015) 'The *Salmonella* effector protein SifA plays a dual role in virulence', *Scientific Reports*. Nature Publishing Group, 5(August). doi: 10.1038/srep12979.

Zhou, D. (1999) 'Role of the *Salmonella* Typhimurium Actin-Binding Protein SipA in Bacterial Internalization', *Science*, 283(5410), pp. 2092–2095. doi: 10.1126/science.283.5410.2092.

Zhou, D. *et al.* (2001) 'A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin

cytoskeleton rearrangements and bacterial internalization', *Molecular Microbiology*, 39(2), pp. 248–259. doi: 10.1046/j.1365-2958.2001.02230.x.

Zhu, W., Smith, J. W. and Huang, C.-M. (2010) 'Mass Spectrometry-Based Label-Free Quantitative Proteomics', *Journal of Biomedicine and Biotechnology*, 2010, pp. 1–6. doi: 10.1155/2010/840518.

Zink, D. L. *et al.* (1980) 'Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica* [27]', *Nature*. doi: 10.1038/283224a0.

Appendix

Raw label free quantitative proteomics data, annotated.

NCBI Identical proteins	Annotation	P value	Fold Change
	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_3541 PE=4 SV=1	0.23986	0.345529382
AceE	Pyruvate dehydrogenase E1 component OS=*Salmonella* typhimurium (strain 4/74) GN=aceE PE=4 SV=1	1.60E-08	0.212805198
AceF	Acetyltransferase component of pyruvate dehydrogenase complex OS=*Salmonella* typhimurium (strain 4/74) GN=aceF PE=3 SV=1	7.92E-06	0.130806513
AckA	Acetate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=ackA PE=3 SV=1	0.01556	0.54267664
AcnB	Aconitate hydratase B OS=*Salmonella* typhimurium (strain 4/74) GN=acnB PE=3 SV=1	0.00256	0.288323782
AdhE	Aldehyde-alcohol dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=adhE PE=3 SV=1	2.83E-05	0.140047541
AdhP	Alcohol dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=adhP PE=3 SV=1	0.00031	0.114238376
arginine decarboxylase	arginine deiminase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=adi SV=1 UP=UP000003971:Chromosome	0.00892	0.301243869
AdK	Adenylate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=adk PE=3 SV=1	7.11E-06	0.391632464
AhpC	Alkyl hydroperoxide reductase subunit C OS=*Salmonella* typhimurium (strain 4/74) GN=ahpC PE=4 SV=1	0.37322	0.376874806
AnsB	L-asparaginase II OS=*Salmonella* typhimurium (strain 4/74) GN=ansB PE=3 SV=1	0.12668	1.647128244
ArtI	arginine ABC transporter ATP-binding protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=artI SV=1 UP=UP000003971:Chromosome	0.00553	0.273692547
Asd	Aspartate-semialdehyde dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=asd PE=3 SV=1	0.1601	0.255414173
AsnC	asparaginyl-tRNA synthetase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=asnC SV=1 UP=UP000003971:Chromosome	0.05971	0.823471641
AspA	Aspartate ammonia-lyase OS=*Salmonella* typhimurium (strain 4/74) GN=aspA PE=3 SV=1	1.24E-05	0.379097257
AspC	Aminotransferase OS=*Salmonella* typhimurium (strain 4/74) GN=aspC PE=3 SV=1	0.00018	0.260504494

AspS	Aspartate--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=aspS PE=3 SV=1	0.12191	0.140239185
AtpA	ATP synthase subunit alpha OS=*Salmonella* typhimurium (strain 4/74) GN=atpA PE=3 SV=1	0.00053	0.303675112
AtpD	ATP synthase subunit beta OS=*Salmonella* typhimurium (strain 4/74) GN=atpD PE=3 SV=1	0.1305	0.369619243
AtpF	ATP synthase subunit b OS=*Salmonella* typhimurium (strain 4/74) GN=atpF PE=3 SV=1	0.01948	0.140413834
AtpG	ATP synthase gamma chain OS=*Salmonella* typhimurium (strain 4/74) GN=atpG PE=3 SV=1	0.14304	0.358398153
AvrA	AvrA OS=*Salmonella* typhimurium (strain 4/74) GN=avrA PE=4 SV=1	0.7261	0.748598805
thioredoxin-dependent thiol peroxidase	Thioredoxin-dependent thiol peroxidase OS=*Salmonella* typhimurium (strain 4/74) GN=bcp PE=4 SV=1	0.00559	2.681504753
bacterioferritin	Bacterioferritin OS=*Salmonella* typhimurium (strain 4/74) GN=bfr PE=3 SV=1	0.01306	0.308255143
BtuE	Thioredoxin/glutathione peroxidase BtuE OS=*Salmonella* typhimurium (strain 4/74) GN=btuE PE=3 SV=1	0.02568	0.17110366
CadA	lysine decarboxylase 1 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=cadA SV=1 UP=UP000003971:Chromosome	5.70E-06	0.038880441
Carbonic anhydrase	Carbonic anhydrase OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_p1066 PE=3 SV=1	0.75659	1.441149763
CheV	Putative chemotaxis signal transduction protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2410 PE=4 SV=1	0.18086	1.569596836
CheW	Purine-binding chemotaxis protein OS=*Salmonella* typhimurium (strain 4/74) GN=cheW PE=4 SV=1	0.0024	4.569573098
ClpB	protein disaggregation chaperone OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=clpB SV=1 UP=UP000003971:Chromosome	0.00021	0.227019661
	ATP-dependent Clp protease proteolytic subunit OS=*Salmonella* typhimurium (strain 4/74) GN=clpP PE=3 SV=1	0.00037	0.241885375
ClpX	ATP-dependent Clp protease ATP-binding subunit ClpX OS=*Salmonella* typhimurium (strain 4/74) GN=clpX PE=3 SV=1	0.95344	1.030377378
Crp	cAMP-regulatory protein OS=*Salmonella* typhimurium (strain 4/74) GN=crp PE=4 SV=1	0.03654	0.459471583
Crr	Glucose-specific PTS system component OS=*Salmonella* typhimurium (strain 4/74) GN=crr PE=4 SV=1	0.03805	0.6444353
CspC	Cold shock-like protein CspC OS=*Salmonella* typhimurium (strain 4/74) GN=cspC PE=4 SV=1	0.00012	0.599962585

CspE	Cold shock-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=cspE PE=4 SV=1	0.00026	0.401657935
CsrA	Carbon storage regulator OS=*Salmonella* typhimurium (strain 4/74) GN=csrA PE=3 SV=1	0.03327	0.136331093
CysK	Cysteine synthase OS=*Salmonella* typhimurium (strain 4/74) GN=cysK PE=3 SV=1	5.67E-06	0.125300039
CysP	Thiosulfate transporter subunit OS=*Salmonella* typhimurium (strain 4/74) GN=cysP PE=4 SV=1	0.33335	0.482026179
4-hydroxy-tetrahydrodipicolinate synthase	4-hydroxy-tetrahydrodipicolinate synthase OS=*Salmonella* typhimurium (strain 4/74) GN=dapA PE=3 SV=1	0.44044	0.20911237
DapD	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=dapD PE=3 SV=1	0.00065	7.746656515
phosphopentomutase	Phosphopentomutase OS=*Salmonella* typhimurium (strain 4/74) GN=deoB PE=3 SV=1	0.12917	4.44074638
purine-nucleoside phosphorylase	purine nucleoside phosphorylase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=deoD SV=1 UP=UP000003971:Chromosome	0.00028	0.021657397
DeoD	Purine nucleoside phosphorylase DeoD-type OS=*Salmonella* typhimurium (strain 4/74) GN=deoD PE=3 SV=1	0.00056	9.103610552
DnaK	Chaperone protein DnaK OS=*Salmonella* typhimurium (strain 4/74) GN=dnaK PE=2 SV=1	0.00122	0.192404222
SteB (type III secretion system effector)	putative dipicolinate reductase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=dpr SV=1 UP=UP000003971:Chromosome	1.07E-05	0.073553053
Dps	DNA protection during starvation protein OS=*Salmonella* typhimurium (strain 4/74) GN=dps PE=3 SV=1	1.62E-06	0.304749222
DsbA	Thiol:disulfide interchange protein OS=*Salmonella* typhimurium (strain 4/74) GN=dsbA PE=3 SV=1	0.08991	0.503700364
EcnB	Entericidin B membrane lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=ecnB PE=4 SV=1	9.62E-05	0.115922238
Eco	Ecotin OS=*Salmonella* typhimurium (strain 4/74) GN=eco PE=3 SV=1	0.01027	0.145697476
Eda	Keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase OS=*Salmonella* typhimurium (strain 4/74) GN=eda PE=4 SV=1	0.01207	0.3974431
Efp	Elongation factor P OS=*Salmonella* typhimurium (strain 4/74) GN=efp PE=3 SV=1	0.00279	0.53885619
Eno	Enolase OS=*Salmonella* typhimurium (strain 4/74) GN=eno PE=3 SV=1	0.00029	0.283517793

FabA	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase OS=*Salmonella* typhimurium (strain 4/74) GN=fabA PE=3 SV=1	4.88E-06	0.212047949
FabB/beta-ketoacyl-[acyl-carrier-protein] synthase I	3-oxoacyl-(Acyl carrier protein) synthase I OS=*Salmonella* typhimurium (strain 4/74) GN=fabB PE=3 SV=1	0.03966	0.188902962
FabD	Malonyl CoA-acyl carrier protein transacylase OS=*Salmonella* typhimurium (strain 4/74) GN=fabD PE=3 SV=1	2.71E-05	0.292487976
FabG	3-oxoacyl-[acyl-carrier-protein] reductase OS=*Salmonella* typhimurium (strain 4/74) GN=fabg PE=4 SV=1	0.00499	0.293241209
enoyl-[acyl-carrier-protein] reductase	enoyl-(acyl carrier protein) reductase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_1814 SV=1 UP=UP000003971:Chromosome	0.00014	0.281598469
FabZ	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ OS=*Salmonella* typhimurium (strain 4/74) GN=fabZ PE=3 SV=1	8.40E-07	6.069717495
FadL	Long-chain fatty acid outer membrane transporter OS=*Salmonella* typhimurium (strain 4/74) GN=fadL PE=4 SV=1	0.21615	3.177487807
Fba	Fructose-bisphosphate aldolase OS=*Salmonella* typhimurium (strain 4/74) GN=fba PE=3 SV=1	0.01024	0.303934316
FbaB	Fructose-bisphosphate aldolase OS=*Salmonella* typhimurium (strain 4/74) GN=fbaB PE=4 SV=1	0.00044	0.227018857
FimA	Type-1 fimbrial protein, A chain OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=fimA SV=1 UP=UP000003971:Chromosome	0.00017	0.159937855
FimA	Type-1 fimbrial protein, A chain OS=*Salmonella* typhimurium (strain 4/74) GN=fimA PE=4 SV=1	8.13E-05	1.32880798
FimH	FimH OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=fimH SV=1 UP=UP000003971:Chromosome	0.3352	0.186881885
FklB	Peptidyl-prolyl cis-trans isomerase OS=*Salmonella* typhimurium (strain 4/74) GN=fklB PE=3 SV=1	0.00011	0.349516868
peptidyl-prolyl cis-trans isomerase	Peptidyl-prolyl cis-trans isomerase OS=*Salmonella* typhimurium (strain 4/74) GN=fkpA PE=3 SV=1	0.00363	11.4900364
FlgB	Flagellar basal body rod protein FlgB OS=*Salmonella* typhimurium (strain 4/74) GN=flgB PE=3 SV=1	0.00025	4.344953243
FlgC	Flagellar basal-body rod protein FlgC OS=*Salmonella* typhimurium (strain 4/74) GN=flgC PE=3 SV=1	0.01233	2.688675884
FlgD	Basal-body rod modification protein FlgD OS=*Salmonella* typhimurium (strain 4/74) GN=flgD PE=3 SV=1	0.0809	1.312326637

FlgE	Flagellar hook protein FlgE OS=*Salmonella* typhimurium (strain 4/74) GN=flgE PE=3 SV=1	0.04783	1.159603776
FlgF	Flagellar basal body protein OS=*Salmonella* typhimurium (strain 4/74) GN=flgF PE=3 SV=1	3.18E-06	1.409549203
FlgG	Flagellar basal-body rod protein FlgG OS=*Salmonella* typhimurium (strain 4/74) GN=flgG PE=3 SV=1	6.91E-05	9.034962356
FlgJ	Flagellar rod assembly protein/muramidase FlgJ OS=*Salmonella* typhimurium (strain 4/74) GN=flgJ PE=4 SV=1	0.01234	3.523196307
FlgK	Flagellar hook-associated protein 1 OS=*Salmonella* typhimurium (strain 4/74) GN=flgK PE=3 SV=1	1	1
FlgL	Flagellar hook-associated protein FlgL OS=*Salmonella* typhimurium (strain 4/74) GN=flgL PE=4 SV=1	1.13E-05	89.62509281
FlgM	Anti-sigma28 factor FlgM OS=*Salmonella* typhimurium (strain 4/74) GN=flgM PE=4 SV=1	0.59253	1.015053336
FliC (mostly Choleraesuis strains)	flagellin OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_2101 SV=1 UP=UP000003971:Chromosome	4.62E-06	3.395549493
FliC	Flagellin OS=*Salmonella* typhimurium (strain 4/74) GN=fliC PE=3 SV=1	6.94E-05	90.70681465
FliD	Flagellar hook-associated protein 2 OS=*Salmonella* typhimurium (strain 4/74) GN=fliD PE=3 SV=1	3.26E-09	0.085421412
FliD/flagellar hook-associated protein 2	flagellar capping protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=fliD SV=1 UP=UP000003971:Chromosome	2.61E-07	0.091112387
FliK	flagellar hook-length control protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=fliK SV=1 UP=UP000003971:Chromosome	0.01496	0.679425327
FliK	Flagellar hook-length control protein OS=*Salmonella* typhimurium (strain 4/74) GN=fliK PE=4 SV=1	0.02704	1.270229024
FliY/putative periplasmic binding transport protein	cystine-binding periplasmic protein precursor OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=fliY SV=1 UP=UP000003971:Chromosome	0.00073	0.407213507
FliC protein (gene = fljB)	Flagellin OS=*Salmonella* typhimurium (strain 4/74) GN=fljB PE=3 SV=1	0.05223	0.102441892
FrdA	Fumarate reductase flavoprotein subunit OS=*Salmonella* typhimurium (strain 4/74) GN=frdA PE=3 SV=1	0.86582	1.199859866
Frr	Ribosome-recycling factor OS=*Salmonella* typhimurium (strain 4/74) GN=frr PE=3 SV=1	0.00096	0.203987128
H-type ferritin	Ferritin OS=*Salmonella* typhimurium (strain 4/74) GN=ftn PE=3 SV=1	0.02031	2.890368376
FtsK/cell division protein, required for cell division and chromosome partitioning	DNA translocase FtsK OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ftsK SV=1 UP=UP000003971:Chromosome	0.22925	0.119248377

FtsZ	Cell division protein FtsZ OS=*Salmonella* typhimurium (strain 4/74) GN=ftsZ PE=3 SV=1	0.28615	0.09139975
FumC	Fumarate hydratase class II OS=*Salmonella* typhimurium (strain 4/74) GN=fumC PE=3 SV=1	0.10779	0.101145658
Fur	Ferric uptake regulation protein OS=*Salmonella* typhimurium (strain 4/74) GN=fur PE=3 SV=1	0.00119	0.370949604
FusA	Elongation factor G OS=*Salmonella* typhimurium (strain 4/74) GN=fusA PE=3 SV=1	0.00021	0.206022675
GalU	UTP--glucose-1-phosphate uridylyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=galU PE=3 SV=1	0.01029	0.435331188
GapA	glyceraldehyde-3-phosphate dehydrogenase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=gapA SV=1 UP=UP000003971:Chromosome	5.40E-07	0.156095713
GarR	2-hydroxy-3-oxopropionate reductase OS=*Salmonella* typhimurium (strain 4/74) GN=garR PE=3 SV=1	0.12496	2.530657549
GcvP	Glycine dehydrogenase (decarboxylating) OS=*Salmonella* typhimurium (strain 4/74) GN=gcvP PE=3 SV=1	0.00607	0.224148067
GcvT	Aminomethyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=gcvT PE=3 SV=1	0.96859	0.993288238
Glu/Leu/Phe/Val dehydrogenase	Glutamate dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1815 PE=3 SV=1	0.23213	0.7153921
GlmM	Phosphoglucosamine mutase OS=*Salmonella* typhimurium (strain 4/74) GN=glmM PE=3 SV=1	5.93E-07	0.053173908
GlmS	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] OS=*Salmonella* typhimurium (strain 4/74) GN=glmS PE=3 SV=1	0.05677	0.223216472
GlnH	Glutamine ABC transporter periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=glnH PE=3 SV=1	0.01894	3.62264865
GlpQ	Glycerophosphodiester phosphodiesterase OS=*Salmonella* typhimurium (strain 4/74) GN=glpQ PE=4 SV=1	0.04494	3.470184725
GltA	Citrate synthase OS=*Salmonella* typhimurium (strain 4/74) GN=gltA PE=3 SV=1	0.5185	1.296551521
glutamate/aspartate ABC transporter substrate-binding protein	Glutamate and aspartate transporter subunit OS=*Salmonella* typhimurium (strain 4/74) GN=gltI PE=4 SV=1	0.19781	1.049169413
GltX	Glutamate--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=gltX PE=3 SV=1	0.29492	0.550634574
GlyA	Serine hydroxymethyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=glyA PE=3 SV=1	7.62E-07	0.089555836

GmhA	Phosphoheptose isomerase OS=*Salmonella* typhimurium (strain 4/74) GN=gmhA PE=3 SV=1	0.00038	0.153988264
Gnd	6-phosphogluconate dehydrogenase, decarboxylating OS=*Salmonella* typhimurium (strain 4/74) GN=gnd PE=3 SV=1	0.00239	0.349964724
GogA	Putative bacteriophage encoded virulence protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2723 PE=4 SV=1	0.17711	0.797971298
GmpA	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=*Salmonella* typhimurium (strain 4/74) GN=gpmA PE=3 SV=1	0.16463	0.899166627
	60 kDa chaperonin OS=*Salmonella* typhimurium (strain 4/74) GN=groL PE=3 SV=1	0.00018	0.110544688
	10 kDa chaperonin OS=*Salmonella* typhimurium (strain 4/74) GN=groS PE=3 SV=1	0.00392	0.12849261
GrpE	Protein GrpE OS=*Salmonella* typhimurium (strain 4/74) GN=grpE PE=3 SV=1	0.00056	0.312291296
GrxA	Glutaredoxin 1 OS=*Salmonella* typhimurium (strain 4/74) GN=grxA PE=4 SV=1	0.81232	0.758663792
GrxB	Glutaredoxin 2 OS=*Salmonella* typhimurium (strain 4/74) GN=grxB PE=4 SV=1	4.58E-05	0.103707534
GrxC	Glutaredoxin 3 OS=*Salmonella* typhimurium (strain 4/74) GN=grxC PE=4 SV=1	0.32658	0.201165363
GstA	Glutathionine S-transferase OS=*Salmonella* typhimurium (strain 4/74) GN=gst PE=4 SV=1	0.00246	5.289746094
GtgA/PipA?	PipA protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1017 PE=4 SV=1	0.00221	0.035215156
head decoration protein	Gifsy-1 prophage head protein gpshp OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=hdpD SV=1 UP=UP000003971:Chromosome	8.98E-06	0.006641817
HisJ	Histidine ABC transporter ATP-binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=hisJ PE=3 SV=1	0.38345	9.431347789
Chaperone protein Skp	Periplasmic chaperone OS=*Salmonella* typhimurium (strain 4/74) GN=hlpA PE=3 SV=1	0.68774	1.092010724
H-NS	DNA-binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=hns PE=3 SV=1	0.00053	0.354403344
HtpG/chaperone Hsp90, heat shock protein C	Chaperone protein HtpG OS=*Salmonella* typhimurium (strain 4/74) GN=htpG PE=3 SV=1	0.00088	0.102022308
HupA	Transcriptional regulator HU subunit alpha OS=*Salmonella* typhimurium (strain 4/74) GN=hupA PE=3 SV=1	0.00013	0.495167104
DNA-binding protein HU-beta	Transcriptional regulator HU subunit beta OS=*Salmonella* typhimurium (strain 4/74) GN=hupB PE=3 SV=1	0.00018	0.511386761
IadA	Isoaspartyl dipeptidase OS=*Salmonella* typhimurium (strain 4/74) GN=iadA PE=3 SV=1	0.18192	0.602519037

NADP-dependent isocitrate dehydrogenase	Isocitrate dehydrogenase [NADP] OS=*Salmonella* typhimurium (strain 4/74) GN=icdA PE=4 SV=1	1.40E-05	12.13546922
IhfA	Integration host factor subunit alpha OS=*Salmonella* typhimurium (strain 4/74) GN=ihfA PE=3 SV=1	0.00016	0.23175541
InfC	Translation initiation factor IF-3 OS=*Salmonella* typhimurium (strain 4/74) GN=infC PE=3 SV=1	0.85572	0.935823908
SpaN	Needle length control protein OS=*Salmonella* typhimurium (strain 4/74) GN=invJ PE=4 SV=1	0.04644	22.70259084
catalase HPII	Catalase OS=*Salmonella* typhimurium (strain 4/74) GN=katE PE=3 SV=1	2.70E-06	0.022078159
catalase-peroxidase	catalase; hydroperoxidase HPI(I) OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=katG SV=1 UP=UP000003971:Chromosome	0.00013	0.072935785
Kbl	2-amino-3-ketobutyrate coenzyme A ligase OS=*Salmonella* typhimurium (strain 4/74) GN=kbl PE=3 SV=1	0.00031	0.35479697
KdgR	Putative transcriptional repressor OS=*Salmonella* typhimurium (strain 4/74) GN=kdgR PE=4 SV=1	0.20277	0.302102238
KdsA	2-dehydro-3-deoxyphosphooctonate aldolase OS=*Salmonella* typhimurium (strain 4/74) GN=kdsA PE=3 SV=1	0.74117	1.075659681
LamB	maltoporin OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=lamB SV=1 UP=UP000003971:Chromosome	0.74261	1.210916393
Lpd	Dihydrolipoyl dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=lpdA PE=4 SV=1	0.35495	1.730876543
Hypothetical protein	Major outer membrane lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=lppA PE=4 SV=1	0.0002	0.072615663
Leucine-responsive regulatory protein	Leucine-responsive transcriptional regulator OS=*Salmonella* typhimurium (strain 4/74) GN=lrp PE=4 SV=1	4.62E-07	12.81219798
LysR	lysR family transcriptional regulator OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=lysR SV=1 UP=UP000003971:Chromosome	0.00058	0.130097102
LysS	Lysine--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=lysS PE=3 SV=1	0.96783	0.974384329
NADP-dependent malic enzyme	Malic enzyme OS=*Salmonella* typhimurium (strain 4/74) GN=maeB PE=4 SV=1	0.03587	2.356891518
MalE	Maltose ABC transporter periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=malE PE=4 SV=1	2.45E-05	0.04284015
MalM	Maltose regulon periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=malM PE=4 SV=1	1.94E-06	0.16894764
Mdh	Malate dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=mdh PE=3 SV=1	0.01633	0.769566713

MetQ	Lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=metQ PE=3 SV=1	0.00221	0.239402726
galactose ABC transporter substrate-binding protein	Galactose transport protein OS=*Salmonella* typhimurium (strain 4/74) GN=mglB PE=4 SV=1	0.00073	4.013693365
MppA	Periplasmic murein peptide-binding protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=mppa SV=1 UP=UP000003971:Chromosome	8.57E-07	0.014476367
MsrA/peptide-methionine (S)-S-oxide reductase	methionine sulfoxide reductase A OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=msrA SV=1 UP=UP000003971:Chromosome	0.00432	0.101513216
NanA	N-acetylneuraminate lyase OS=*Salmonella* typhimurium (strain 4/74) GN=nanA PE=3 SV=1	0.00723	3.407863755
Ndk	Nucleoside diphosphate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=ndk PE=3 SV=1	0.0922	0.633487567
NemA	N-ethylmaleimide reductase OS=*Salmonella* typhimurium (strain 4/74) GN=nemA PE=4 SV=1	0.00406	0.337748892
PhoE/Phosphoporin	Putative outer membrane porin OS=*Salmonella* typhimurium (strain 4/74) GN=nmpC PE=3 SV=1	4.23E-05	0.372412626
NrdB	Ribonucleotide-diphosphate reductase subunit beta OS=*Salmonella* typhimurium (strain 4/74) GN=nrdB PE=4 SV=1	0.14294	0.092862931
NusA	Transcription termination/antitermination protein NusA OS=*Salmonella* typhimurium (strain 4/74) GN=nusA PE=3 SV=1	0.72381	0.77880257
NusB	N utilization substance protein B homolog OS=*Salmonella* typhimurium (strain 4/74) GN=nusB PE=3 SV=1	0.00093	0.231382144
OmpA	Outer membrane protein A OS=*Salmonella* typhimurium (strain 4/74) GN=ompA PE=3 SV=1	1.61E-06	0.119960275
PhoE/Phosphoporin	Outer membrane porin protein C OS=*Salmonella* typhimurium (strain 4/74) GN=ompC PE=3 SV=1	7.76E-06	0.25112902
OmpF	Outer membrane protein F OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ompf SV=1 UP=UP000003971:Chromosome	0.00031	0.187994282
OmpW	Outer membrane protein W OS=*Salmonella* typhimurium (strain 4/74) GN=ompW PE=4 SV=1	0.04985	2.2594644
OmpX	Outer membrane protein X OS=*Salmonella* typhimurium (strain 4/74) GN=ompX PE=4 SV=1	5.14E-07	0.143839621
OrgC	Putative cytoplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=orgC PE=4 SV=1	0.21788	0.028481798

Ornithine carbamoyltransferase	Ornithine carbamoyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_4662 PE=3 SV=1	0.65796	1.347727462
OsmC family peroxiredoxin	Osmotically inducible protein C OS=*Salmonella* typhimurium (strain 4/74) GN=osmC PE=4 SV=1	0.18408	0.332039256
OsmE	DNA-binding transcriptional activator OsmE OS=*Salmonella* typhimurium (strain 4/74) GN=osmE PE=4 SV=1	0.00132	0.087607397
OsmY	Periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_4766 PE=4 SV=1	1.83E-07	0.473391916
PagC	Virulence membrane protein PagC OS=*Salmonella* typhimurium (strain 4/74) GN=pagC PE=4 SV=1	1.04E-06	0.307424062
Pal/peptidoglycan-associated lipoprotein precursor	Peptidoglycan-associated lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=pal PE=3 SV=1	4.40E-05	0.129794336
PckA	Phosphoenolpyruvate carboxykinase (ATP) OS=*Salmonella* typhimurium (strain 4/74) GN=pckA PE=3 SV=1	0.08111	0.763851998
cytosol nonspecific dipeptidase	Aminoacyl-histidine dipeptidase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=pepd SV=1 UP=UP000003971:Chromosome	0.00236	0.009195 23
cytosol nonspecific dipeptidase	Aminoacyl-histidine dipeptidase OS=*Salmonella* typhimurium (strain 4/74) GN=pepD PE=4 SV=1	0.00036	3.511031363
PepN	Aminopeptidase N OS=*Salmonella* typhimurium (strain 4/74) GN=pepN PE=4 SV=1	0.09602	0.439915513
	Xaa-Pro dipeptidase OS=*Salmonella* typhimurium (strain 4/74) GN=pepQ PE=3 SV=1	0.00018	0.160967197
PepT	Peptidase T OS=*Salmonella* typhimurium (strain 4/74) GN=pepT PE=3 SV=1	0.00269	0.186846191
DNA packaging protein	DNA packaging-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2711 PE=4 SV=1	0.15875	1.477118568
PflB	Formate acetyltransferase 1 OS=*Salmonella* typhimurium (strain 4/74) GN=pflB PE=4 SV=1	0.16905	0.783463557
Pgi	glucose-6-phosphate isomerase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=pgi SV=1 UP=UP000003971:Chromosome	0.36032	0.114582 29
PgK	Phosphoglycerate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=pgk PE=3 SV=1	1.79E-05	0.293989192
Pgm	Phosphoglucomutase OS=*Salmonella* typhimurium (strain 4/74) GN=pgm PE=3 SV=1	0.0039	0.198705615
Phage major capsid protein	Major capsid protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2051 PE=4 SV=1	0.66639	1.293975657
Phage tail assembly protein	Sb15 OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2043 PE=4 SV=1	0.3697	1.46592075

PhoN/phosphatase PAP2 family protein	Acid phosphatase OS=*Salmonella* typhimurium (strain 4/74) GN=phoN PE=3 SV=1	9.25E-05	0.198016164
PhoP	DNA-binding transcriptional regulator PhoP OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=phoP SV=1 UP=UP000003971:Chromosome	1.86E-05	0.000842021
PhoP	DNA-binding transcriptional regulator PhoP OS=*Salmonella* typhimurium (strain 4/74) GN=phoP PE=4 SV=1	2.77E-05	10.08137268
PipB	pathogenicity island encoded protein: SPI3 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=pipB SV=1 UP=UP000003971:Chromosome	0.0437	0.155003015
PipB2	Effector protein pipB2 OS=*Salmonella* typhimurium (strain 4/74) GN=pipB2 PE=4 SV=1	0.01793	0.754266478
NONE	phosphoglyceromutase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=pmgI SV=1 UP=UP000003971:Chromosome	0.64234	0.715401133
PmgI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase OS=*Salmonella* typhimurium (strain 4/74) GN=pmgI PE=3 SV=1	0.57781	1.882056132
Pnp	Polyribonucleotide nucleotidyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=pnp PE=3 SV=1	0.00038	0.166098406
	Putrescine-binding periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=potD PE=3 SV=1	4.84E-05	0.285922345
Ppa	Inorganic pyrophosphatase OS=*Salmonella* typhimurium (strain 4/74) GN=ppa PE=3 SV=1	0.0005	0.401126175
PpiB	Peptidyl-prolyl cis-trans isomerase OS=*Salmonella* typhimurium (strain 4/74) GN=ppiB PE=3 SV=1	0.00666	0.542833221
Pps	Phosphoenolpyruvate synthase OS=*Salmonella* typhimurium (strain 4/74) GN=pps PE=3 SV=1	0.0902	0.211989202
PrgI	Needle complex major subunit OS=*Salmonella* typhimurium (strain 4/74) GN=prgI PE=4 SV=1	8.54E-07	6.052471615
PrgJ	Needle complex minor subunit OS=*Salmonella* typhimurium (strain 4/74) GN=prgJ PE=4 SV=1	0.00028	3.311588211
oligopeptidase A	Oligopeptidase A OS=*Salmonella* typhimurium (strain 4/74) GN=prlC PE=3 SV=1	0.7361	1.071216851
ProS	Proline--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=proS PE=3 SV=1	0.2018	0.659101285
ribose-phosphate pyrophosphokinase	Ribose-phosphate pyrophosphokinase OS=*Salmonella* typhimurium (strain 4/74) GN=prsA PE=3 SV=1	0.00093	0.2915857
PstS	phosphate ABC transporter periplasmic substrate-binding protein PstS OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=pstS SV=1 UP=UP000003971:Chromosome	6.81E-07	0.017992985

PstS	Phosphate-binding protein PstS OS=*Salmonella* typhimurium (strain 4/74) GN=pstS PE=3 SV=1	0.07547	1.928986584
Pta	Phosphate acetyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=pta PE=3 SV=1	0.00227	0.118038819
PtsL	Phosphoenolpyruvate-protein phosphotransferase OS=*Salmonella* typhimurium (strain 4/74) GN=ptsI PE=3 SV=1	0.1804	0.372500396
adenylosuccinate synthetase	Adenylosuccinate synthetase OS=*Salmonella* typhimurium (strain 4/74) GN=purA PE=3 SV=1	0.84254	1.059064623
PurB	Adenylosuccinate lyase OS=*Salmonella* typhimurium (strain 4/74) GN=purB PE=3 SV=1	0.19925	0.511967461
PykA	Pyruvate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=pykA PE=3 SV=1	0.00022	0.130296299
pykF/pyruvate kinase I	Pyruvate kinase OS=*Salmonella* typhimurium (strain 4/74) GN=pykF PE=3 SV=1	0.07106	0.228690051
RbfA	Ribosome-binding factor A OS=*Salmonella* typhimurium (strain 4/74) GN=rbfA PE=3 SV=1	0.03576	0.458134992
RbsB	D-ribose transporter subunit RbsB OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=rbsB SV=1 UP=UP000003971:Chromosome	0.00242	0.659082249
RecA	Protein RecA OS=*Salmonella* typhimurium (strain 4/74) GN=recA PE=3 SV=1	0.00051	0.041223682
RfbH (LPS biosynthesis protein)	CDP-6-deoxy-D-xylo-4-hexulose-3-dehydrase OS=*Salmonella* typhimurium (strain 4/74) GN=rfbH PE=3 SV=1	0.33893	1.745190281
RibB	3,4-dihydroxy-2-butanone 4-phosphate synthase OS=*Salmonella* typhimurium (strain 4/74) GN=ribB PE=3 SV=1	0.0006	0.32224327
RibH	6,7-dimethyl-8-ribityllumazine synthase OS=*Salmonella* typhimurium (strain 4/74) GN=ribH PE=3 SV=1	0.07249	0.173936427
RihA	Pyrimidine-specific ribonucleoside hydrolase RihA OS=*Salmonella* typhimurium (strain 4/74) GN=rihA PE=3 SV=1	0.0259	0.089067947
RmuC	DNA stabilization protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0384 SV=1 UP=UP000003971:Chromosome	0.00024	0.137992374
RpiA	Ribose-5-phosphate isomerase A OS=*Salmonella* typhimurium (strain 4/74) GN=rpiA PE=3 SV=1	0.0493	0.362767443
RplA	50S ribosomal protein L1 OS=*Salmonella* typhimurium (strain 4/74) GN=rplA PE=3 SV=1	1.08E-05	0.320063464
RplB	50S ribosomal protein L2 OS=*Salmonella* typhimurium (strain 4/74) GN=rplB PE=3 SV=1	0.00363	0.108364727
RplC	50S ribosomal protein L3 OS=*Salmonella* typhimurium (strain 4/74) GN=rplC PE=3 SV=1	1.89E-05	0.127800322
RplD	50S ribosomal protein L4 OS=*Salmonella* typhimurium (strain 4/74) GN=rplD PE=3 SV=1	0.09905	0.24688568
RplE	50S ribosomal protein L5 OS=*Salmonella* typhimurium (strain 4/74) GN=rplE PE=3 SV=1	0.00108	0.232623563

RplF	50S ribosomal protein L6 OS=*Salmonella* typhimurium (strain 4/74) GN=rplF PE=3 SV=1	2.18E-05	0.16665024
RplI	50S ribosomal protein L9 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_4563 SV=1 UP=UP000003971:Chromosome	0.00056	0.451126651
RplJ	50S ribosomal protein L10 OS=*Salmonella* typhimurium (strain 4/74) GN=rplJ PE=3 SV=1	6.35E-07	0.275624183
RplK	50S ribosomal protein L11 OS=*Salmonella* typhimurium (strain 4/74) GN=rplK PE=3 SV=1	9.99E-07	0.25071013
RplL	50S ribosomal protein L7/L12 OS=*Salmonella* typhimurium (strain 4/74) GN=rplL PE=3 SV=1	0.00068	0.496985315
RplM	50S ribosomal protein L13 OS=*Salmonella* typhimurium (strain 4/74) GN=rplM PE=3 SV=1	5.64E-06	0.346535983
RplN	50S ribosomal protein L14 OS=*Salmonella* typhimurium (strain 4/74) GN=rplN PE=3 SV=1	1.77E-05	0.105391949
RplO	50S ribosomal protein L15 OS=*Salmonella* typhimurium (strain 4/74) GN=rplO PE=3 SV=1	0.09561	0.416760021
RplQ	50S ribosomal protein L17 OS=*Salmonella* typhimurium (strain 4/74) GN=rplQ PE=3 SV=1	0.03242	0.505023339
RplR	50S ribosomal protein L18 OS=*Salmonella* typhimurium (strain 4/74) GN=rplR PE=3 SV=1	1.30E-05	0.195282112
RplS	50S ribosomal protein L19 OS=*Salmonella* typhimurium (strain 4/74) GN=rplS PE=3 SV=1	0.21698	0.29487428
RplT	50S ribosomal protein L20 OS=*Salmonella* typhimurium (strain 4/74) GN=rplT PE=3 SV=1	0.01031	0.170646337
RplU	50S ribosomal protein L21 OS=*Salmonella* typhimurium (strain 4/74) GN=rplU PE=3 SV=1	8.63E-05	0.233324164
RplV	50S ribosomal protein L22 OS=*Salmonella* typhimurium (strain 4/74) GN=rplV PE=3 SV=1	0.38446	1.385857496
RplW	50S ribosomal protein L23 OS=*Salmonella* typhimurium (strain 4/74) GN=rplW PE=3 SV=1	0.03237	0.545707511
RplX	50S ribosomal protein L24 OS=*Salmonella* typhimurium (strain 4/74) GN=rplX PE=3 SV=1	9.05E-05	0.477111654
RpmB	50S ribosomal protein L28 OS=*Salmonella* typhimurium (strain 4/74) GN=rpmB PE=3 SV=1	7.99E-05	0.489799108
RpmD	50S ribosomal protein L30 OS=*Salmonella* typhimurium (strain 4/74) GN=rpmD PE=3 SV=1	0.10345	0.659418759
RpmI	50S ribosomal protein L35 OS=*Salmonella* typhimurium (strain 4/74) GN=rpmI PE=3 SV=1	0.09014	0.071020583
DNA-directed RNA polymerase subunit alpha	DNA-directed RNA polymerase subunit alpha OS=*Salmonella* typhimurium (strain 4/74) GN=rpoA PE=3 SV=1	0.00424	0.235089556
RpoB	DNA-directed RNA polymerase subunit beta OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=rpoB SV=1 UP=UP000003971:Chromosome	0.01771	0.435377844
RpoC	DNA-directed RNA polymerase subunit beta' OS=*Salmonella* typhimurium (strain 4/74) GN=rpoC PE=3 SV=1	0.09544	0.336417441

RpoZ	DNA-directed RNA polymerase subunit omega OS=*Salmonella* typhimurium (strain 4/74) GN=rpoZ PE=3 SV=1	0.19322	0.403426376
RpsB	30S ribosomal protein S2 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsB PE=3 SV=1	0.9978	1.000570636
RpsC	30S ribosomal protein S3 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsC PE=3 SV=1	4.50E-05	0.099535053
RpsD	30S ribosomal protein S4 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsD PE=3 SV=1	0.00241	0.174613797
RpsE	30S ribosomal protein S5 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsE PE=3 SV=1	0.0053	0.367105759
RpsF	30S ribosomal protein S6 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsF PE=3 SV=1	5.18E-05	0.337261698
RpsG	30S ribosomal protein S7 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsG PE=3 SV=1	0.48057	0.794388223
RspH	30S ribosomal protein S8 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsH PE=3 SV=1	0.00696	0.288400612
RpsI	30S ribosomal protein S9 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsI PE=3 SV=1	0.00139	0.182837877
RspJ	30S ribosomal protein S10 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsJ PE=3 SV=1	3.81E-05	0.063127011
RpsK	30S ribosomal protein S11 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsK PE=3 SV=1	0.03477	0.220927758
RpsM	30S ribosomal protein S13 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsM PE=3 SV=1	0.00028	0.159471073
RpsO	30S ribosomal protein S15 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsO PE=3 SV=1	0.00127	0.642809938
RpsP	30S ribosomal protein S16 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsP PE=3 SV=1	0.78149	1.027396 46
RpsQ	30S ribosomal protein S17 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsQ PE=3 SV=1	0.16402	0.496305952
RpsR	30S ribosomal protein S18 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsR PE=3 SV=1	0.00418	0.374939543
RpsS	30S ribosomal protein S19 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsS PE=3 SV=1	0.039	0.129666787
RpsT	30S ribosomal protein S20 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsT PE=3 SV=1	5.11E-05	0.260554965
RspA/D-galactonate dehydratase family protein	30S ribosomal protein S1 OS=*Salmonella* typhimurium (strain 4/74) GN=rpsA PE=3 SV=1	0.00059	0.138983844

Hypothetical protein	hypothetical protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0346 SV=1 UP=UP000003971:Chromosome	2.28E-05	0.005021711
Scaffolding protien	scaffolding protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0381 SV=1 UP=UP000003971:Chromosome	0.00096	0.082482256
Coat protein	coat protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0382 SV=1 UP=UP000003971:Chromosome	1.76E-07	0.001107555
Hypothetical protein/DNA transfer protein	DNA transfer protein gp7 precursor OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0388 SV=1 UP=UP000003971:Chromosome	0.02939	0.457224777
DNA transfer protein	DNA transfer protein gp20 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0389 SV=1 UP=UP000003971:Chromosome	1.03E-06	0.038732984
Hypothetical protein	injection protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0390 SV=1 UP=UP000003971:Chromosome	0.00032	0.066582787
Hypothetical protein	hypothetical protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_0392 SV=1 UP=UP000003971:Chromosome	1.38E-06	0.032736186
NONE	lysozyme OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_1065 SV=1 UP=UP000003971:Chromosome	0.00013	3.461724624
DUF2303 domain-containing protein	hypothetical protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_1281 SV=1 UP=UP000003971:Chromosome	8.56E-07	0.030065112
NONE	Phage tail assembly chaperone gp38 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_1327 SV=1 UP=UP000003971:Chromosome	0.1025	0.037378775
putative inner membrane protein	putative inner membrane protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_2891 SV=1 UP=UP000003971:Chromosome	0.00043	0.671209154
peptidyl-prolyl cis-trans isomerase	FKBP-type peptidyl-prolyl cis-trans isomerase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=SCA50_3605 SV=1 UP=UP000003971:Chromosome	0.00035	0.003733973
putative glucosamine-fructose-6-	putative glucosamine-fructose-6-phosphate aminotransferase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str.	0.38401	0.537877 67

phosphate aminotransferase	SCSA50 GN=SCA50_4697 SV=1 UP=UP000003971:Chromosome		
SecB	Protein-export protein SecB OS=*Salmonella* typhimurium (strain 4/74) GN=secB PE=3 SV=1	0.00932	0.548745283
serine--tRNA ligase	Serine--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=serS PE=3 SV=1	0.0003	0.169176324
SifA	Secreted effector protein OS=*Salmonella* typhimurium (strain 4/74) GN=sifA PE=4 SV=1	1.71E-07	0.234631189
Ig-like domain repeat protein	Putative inner membrane protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_4456 PE=4 SV=1	2.00E-05	0.564745854
SipA	Secreted effector protein OS=*Salmonella* typhimurium (strain 4/74) GN=sipA PE=4 SV=1	2.86E-07	4.631745236
SipB	cell invasion protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=sipB SV=1 UP=UP000003971:Chromosome	2.87E-05	0.055299343
SipB	Cell invasion protein OS=*Salmonella* typhimurium (strain 4/74) GN=sipB PE=4 SV=1	6.37E-06	13.7026342
SipC	Translocation machinery component OS=*Salmonella* typhimurium (strain 4/74) GN=sipC PE=4 SV=1	0.00455	2.438775414
SipD	cell invasion protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=sipD SV=1 UP=UP000003971:Chromosome	0.00055	0.002828418
SipD	Translocation machinery component OS=*Salmonella* typhimurium (strain 4/74) GN=sipD PE=4 SV=1	4.24E-09	193.7745916
SlrP	leucine-rich repeat-containing protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=slrP SV=1 UP=UP000003971:Chromosome	3.01E-05	0.014640115
SlrP	Leucine-rich repeat-containing protein OS=*Salmonella* typhimurium (strain 4/74) GN=slrP PE=4 SV=1	0.20912	1.683214845
SlyA	Transcriptional regulator SlyA OS=*Salmonella* typhimurium (strain 4/74) GN=slyA PE=3 SV=1	0.00758	0.316122319
SlyB/Hypothetical protein	Outer membrane lipoprotein SlyB OS=*Salmonella* typhimurium (strain 4/74) GN=slyB PE=4 SV=1	0.00185	0.328198984
SodB	Superoxide dismutase OS=*Salmonella* typhimurium (strain 4/74) GN=sodB PE=3 SV=1	0.00013	0.308751547
SodC1	Superoxide dismutase [Cu-Zn] 1 OS=*Salmonella* typhimurium (strain 4/74) GN=sodC1 PE=3 SV=1	0.00551	0.276802083
SopA	Secreted effector protein OS=*Salmonella* typhimurium (strain 4/74) GN=sopA PE=4 SV=1	5.16E-05	14.72281781
NONE	Secreted effector protein OS=*Salmonella* typhimurium (strain 4/74) GN=sopB PE=4 SV=1	1.12E-06	0.287373003

SopD2	Secreted effector protein sopD2 OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=sopD SV=1 UP=UP000003971:Chromosome	9.84E-05	0.056682172
SopD	Sop effector protein SopD OS=*Salmonella* typhimurium (strain 4/74) GN=sopD PE=4 SV=1	0.66077	0.704520166
SopD2	SopD-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_0958 PE=4 SV=1	0.01768	5.708757594
SopE	Invasion-associated secreted protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2826 PE=4 SV=1	2.67E-07	194.2496243
SopE2	TypeIII-secreted protein effector: invasion-associated protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=sopE2 SV=1 UP=UP000003971:Chromosome	0.02102	0.001958247
SopE2	Type III-secreted effector protein OS=*Salmonella* typhimurium (strain 4/74) GN=sopE2 PE=4 SV=1	1.88E-05	10.63217756
SpaN	Surface presentation of antigens protein spaN OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=span SV=1 UP=UP000003971:Chromosome	0.02648	0.258531464
SptP	Protein tyrosine phosphatase/GTPase activating protein OS=*Salmonella* typhimurium (strain 4/74) GN=sptP PE=4 SV=1	3.74E-05	8.42413171
SpvC	virulence protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=spvC SV=1 UP=UP000003971:Plasmid pSCV50	3.48E-07	0.011639625
Ssb	ssDNA-binding protein controls activity of RecBCD nuclease OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ssb SV=1 UP=UP000003971:Chromosome	0.00172	0.041986476
SsB	Single-stranded DNA-binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=ssb PE=3 SV=1	2.88E-05	0.199250433
SseL	Deubiquitinase OS=*Salmonella* typhimurium (strain 4/74) GN=sseL PE=4 SV=1	0.00183	4.762751794
SspA	Stringent starvation protein A OS=*Salmonella* typhimurium (strain 4/74) GN=sspA PE=3 SV=1	0.29895	0.551363992
SteA/Hypothetical protein	Secreted effector protein steA OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=stea SV=1 UP=UP000003971:Chromosome	0.00171	0.018975561
SteA	Putative cytoplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1596 PE=4 SV=1	0.00015	7.189856254
Tail protein	side tail fiber protein OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=stf SV=1 UP=UP000003971:Chromosome	6.65E-06	0.036572021

peroxiredoxin	Peroxidase OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_0421 PE=4 SV=1	0.39832	0.407953227
NONE	ATP-dependent Clp protease proteolytic subunit OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1024 PE=3 SV=1	0.01287	2.129331246
Hypothetical protein	Gifsy-2 prophage protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1047 PE=4 SV=1	0.07639	0.568664977
DUF3626 domain-containing protein	Putative cytoplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1237 PE=4 SV=1	2.51E-06	10.68875885
ABC transporter substrate-binding protein	Putative ABC transporter periplasmic binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1255 PE=4 SV=1	0.00192	2.229181995
DUF2219 domain-containing protein	Putative outer membrane protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1333 PE=4 SV=1	0.00339	3.486492349
NONE	Putative inner membrane protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1711 PE=4 SV=1	5.54E-06	0.183249787
Tail sheath protein	Tail Sheath protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2045 PE=4 SV=1	0.00803	4.600139611
Hypothetical protein	Uncharacterized protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2071 PE=4 SV=1	0.00013	0.214686016
DUF2303 domain-containing protein	Putative phage protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2074 PE=4 SV=1	0.00859	2.699897978
phage minor tail protein G	Minor tail-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2705 PE=4 SV=1	0.00181	0.077626226
Tail protein	Gifsy-1 prophage VmtV OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2706 PE=4 SV=1	2.52E-05	0.031281153
minor capsid protein E	Phage head-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2712 PE=4 SV=1	3.21E-06	0.026498692
Head decoration protein	Phage head-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2713 PE=4 SV=1	0.01566	1.647722689
S49 family peptidase	Head-tail preconnector-like protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2714 PE=4 SV=1	6.51E-07	0.076301839
LysR family transcriptional regulator	Putative transcriptional regulator OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_3052 PE=4 SV=1	0.21121	2.033859913
Lipoprotein	DcrB protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_3747 PE=4 SV=1	0.08336	0.114101539
rhodanese-like domain-containing protein	Putative secreted protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_3876 PE=4 SV=1	0.01824	0.094424626
NAD-dependent succinate-semialdehyde dehydrogenase	Putative NAD-dependent aldehyde dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_4723 PE=3 SV=1	0.00016	7.30870617

	Aminoglycoside resistance protein A OS=*Salmonella* typhimurium (strain 4/74) GN=strA PE=3 SV=1	0.10635	2.441751557
SucB	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OS=*Salmonella* typhimurium (strain 4/74) GN=sucB PE=3 SV=1	4.72E-07	0.209240054
SucC	succinyl-CoA synthetase subunit beta OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=sucC SV=1 UP=UP000003971:Chromosome	0.00052	0.262556916
SucD	Succinate--CoA ligase [ADP-forming] subunit alpha OS=*Salmonella* typhimurium (strain 4/74) GN=sucD PE=3 SV=1	0.01675	0.26968723
inositol monophosphatase	Inositol monophosphatase OS=*Salmonella* typhimurium (strain 4/74) GN=suhB PE=4 SV=1	0.41565	1.483628143
SurA	peptidyl-prolyl cis-trans isomerase SurA OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=surA SV=1 UP=UP000003971:Chromosome	0.00032	0.302708447
TalB	Transaldolase OS=*Salmonella* typhimurium (strain 4/74) GN=talB PE=3 SV=1	2.77E-05	0.293097493
tripartite tricarboxylate transporter substrate binding protein	Tricarboxylic transport OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_2920 PE=4 SV=1	0.32689	2.543523172
Tdh	L-threonine 3-dehydrogenase OS=*Salmonella* typhimurium (strain 4/74) GN=tdh PE=3 SV=1	0.00011	0.289171837
ThrS	Threonine--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=thrS PE=3 SV=1	0.19854	0.223311665
Tig	Trigger factor OS=*Salmonella* typhimurium (strain 4/74) GN=tig PE=3 SV=1	0.00087	0.287960589
TktA	Transketolase OS=*Salmonella* typhimurium (strain 4/74) GN=tktA PE=3 SV=1	0.00069	0.356762372
TolB	translocation protein TolB OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=tolB SV=1 UP=UP000003971:Chromosome	3.82E-05	0.190589606
TpiA	Triosephosphate isomerase OS=*Salmonella* typhimurium (strain 4/74) GN=tpiA PE=3 SV=1	0.05117	0.60925175
Tpx	Probable thiol peroxidase OS=*Salmonella* typhimurium (strain 4/74) GN=tpx PE=3 SV=1	0.00012	0.539653857
TrxA	Thioredoxin OS=*Salmonella* typhimurium (strain 4/74) GN=trxA PE=3 SV=1	0.06001	0.569553586
Tsf/Translation elongation factor Ts	elongation factor Ts OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=tsf SV=1 UP=UP000003971:Chromosome	0.00055	0.316929064
Tsx	nucleoside channel; receptor of phage T6 and colicin K OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=tsx SV=1 UP=UP000003971:Chromosome	0.61378	0.838212902

Elongation factor Tu	Elongation factor Tu OS=*Salmonella* typhimurium (strain 4/74) GN=tuf PE=3 SV=1	0.00289	0.11399221
TypA	GTP-binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=typA PE=4 SV=1	7.73E-05	0.028689541
TyrS	Tyrosine--tRNA ligase OS=*Salmonella* typhimurium (strain 4/74) GN=tyrS PE=3 SV=1	0.00244	0.193751118
Udp	Uridine phosphorylase OS=*Salmonella* typhimurium (strain 4/74) GN=udp PE=3 SV=1	0.55754	0.709608141
UgpB	Glycerol-3-phosphate transporter periplasmic binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=ugpB PE=4 SV=1	0.09352	0.522632056
Universal stress protein F	Universal stress protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1664 PE=3 SV=1	0.25852	1.496701876
Upp	Uracil phosphoribosyltransferase OS=*Salmonella* typhimurium (strain 4/74) GN=upp PE=3 SV=1	0.97386	1.017904829
UshA	Protein UshA OS=*Salmonella* typhimurium (strain 4/74) GN=ushA PE=3 SV=1	0.22594	0.361995685
phage portal protein	Gifsy-2 prophage VmtV OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=vmtV SV=1 UP=UP000003971:Chromosome	0.539	0.42135284
WraB/TrpR binding protein	NAD(P)H dehydrogenase (quinone) OS=*Salmonella* typhimurium (strain 4/74) GN=wraB PE=3 SV=1	0.12641	0.086433854
YadF	Carbonic anhydrase OS=*Salmonella* typhimurium (strain 4/74) GN=yadF PE=3 SV=1	1.43E-05	0.196200856
YajQ	UPF0234 protein YajQ OS=*Salmonella* typhimurium (strain 4/74) GN=yajQ PE=3 SV=1	0.00478	0.521035007
YbaY	Uncharacterized lipoprotein ybaY OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ybay SV=1 UP=UP000003971:Chromosome	1.72E-05	0.037819687
YbbN	Thioredoxin domain protein OS=*Salmonella* typhimurium (strain 4/74) GN=ybbN PE=4 SV=1	0.67008	0.588260851
Hypothetical protein/dinuclear metal center protein, YbgI family	GTP cyclohydrolase 1 type 2 homolog OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_0733 PE=3 SV=1	0.37164	0.224326916
YbiB	Uncharacterized protein ybiB OS=*Salmonella* typhimurium (strain 4/74) GN=ybib PE=4 SV=1	0.90651	0.968674963
YbiS	Putative L,D-transpeptidase YbiS OS=*Salmonella* typhimurium (strain 4/74) GN=ybis PE=4 SV=1	0.23209	0.753797421
Lipoprotein	Putative lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=ybjP PE=4 SV=1	4.07E-06	0.076081955
YcbK (DUF882 domain-containing protein)	Putative outer membrane protein OS=*Salmonella* typhimurium (strain 4/74) GN=ycbK PE=4 SV=1	0.23899	1.655779209

DUF1471 domain-containing protein	Protein ydgH OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ydgh SV=1 UP=UP000003971:Chromosome	0.02369	0.37717572
YdhD	Glutaredoxin OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1440 PE=3 SV=1	0.39236	0.717271762
NAD(P)H nitroreductase	Nitroreductase family protein OS=*Salmonella* typhimurium (strain 4/74) GN=STM474_1300 PE=4 SV=1	0.18041	0.478526304
YeaD	Aldose 1-epimerase OS=*Salmonella* typhimurium (strain 4/74) GN=yeaD PE=4 SV=1	0.34564	2.405130963
YebC/YebC/PmpR family DNA-binding transcriptional regulator	Probable transcriptional regulatory protein YebC OS=*Salmonella* typhimurium (strain 4/74) GN=yebC PE=3 SV=1	0.01428	0.15842031
ABC transporter substrate-binding protein	Putative transport protein OS=*Salmonella* typhimurium (strain 4/74) GN=yehZ PE=4 SV=1	6.90E-06	0.333642072
RaiA	Translation inhibitor protein RaiA OS=*Salmonella* typhimurium (strain 4/74) GN=yfiA PE=4 SV=1	7.98E-06	0.203916696
GrcA	Autonomous glycyl radical cofactor OS=*Salmonella* typhimurium (strain 4/74) GN=yfiD PE=3 SV=1	0.15639	0.819023704
BamD/outer membrane protein assembly factor BamD	Outer membrane protein assembly factor BamD OS=*Salmonella* typhimurium (strain 4/74) GN=yfiO PE=3 SV=1	0.00147	0.079264862
YgaM/hypothetical	YgaM protein OS=*Salmonella* typhimurium (strain 4/74) GN=ygaM PE=4 SV=1	5.95E-05	0.090201136
YghA/SDR family NAD(P)-dependent oxidoreductase	Oxidoreductase OS=*Salmonella* typhimurium (strain 4/74) GN=yghA PE=4 SV=1	0.0018	0.149126005
gfo/Idh/MocA family oxidoreductase	putative dehydrogenase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=ygjR SV=1 UP=UP000003971:Chromosome	3.80E-07	0.055308439
Sigma cross-reacting protein 27A/Enhancing lycopene biosynthesis protein 2	Glyoxalase OS=*Salmonella* typhimurium (strain 4/74) GN=yhbL PE=3 SV=1	0.27012	2.548621603
NfuA	Fe/S biogenesis protein NfuA OS=*Salmonella* typhimurium (strain 4/74) GN=yhgI PE=3 SV=1	0.11803	0.466858128
YicC family protein	UPF0701 protein yicC OS=*Salmonella* typhimurium (strain 4/74) GN=yicc PE=4 SV=1	0.00261	0.276520154
YifE/DUF413 domain-containing protein	Protein yifE OS=*Salmonella* typhimurium (strain 4/74) GN=yifE PE=4 SV=1	0.00927	0.139578919
YjeI/DUF4156 domain-containing protein	Putative outer membrane lipoprotein OS=*Salmonella* typhimurium (strain 4/74) GN=yjeI PE=4 SV=1	0.10044	0.049416017

YjgF	Putative translation initiation inhibitor OS=*Salmonella* typhimurium (strain 4/74) GN=yjgF PE=4 SV=1	7.70E-05	0.358643986
EttA (energy-dependent translational throttle protein)	Putative ABC transporter ATP-binding protein OS=*Salmonella* typhimurium (strain 4/74) GN=yjjK PE=4 SV=1	0.0003	0.074847109
LsrB/Hypothetical protein	Putative sugar transport protein OS=*Salmonella* typhimurium (strain 4/74) GN=yneA PE=4 SV=1	0.02836	0.496668483
LsrF/3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase	3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase OS=*Salmonella* typhimurium (strain 4/74) GN=yneB PE=3 SV=1	6.98E-05	0.496292512
NADH-dependent alcohol dehydrogenase	putative alcohol dehydrogenase OS=*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SCSA50 GN=yqhD SV=1 UP=UP000003971:Chromosome	1.58E-06	0.076764979
DUF1090 domain-containing protein	Putative periplasmic protein OS=*Salmonella* typhimurium (strain 4/74) GN=yqjC PE=4 SV=1	0.09887	0.38720275
DUF883 domain-containing protein	Putative inner membrane protein OS=*Salmonella* typhimurium (strain 4/74) GN=yqjD PE=4 SV=1	2.64E-06	0.088837824
MlaC	Putative transport protein OS=*Salmonella* typhimurium (strain 4/74) GN=yrbC PE=4 SV=1	1.75E-05	0.160283477