Characterisation of σ^E-regulated stress response proteins and their contributions to *Salmonella* physiology

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Abstract

The ability of *Salmonella* to sense and respond to changing environmental conditions is central to its survival within a host and the food chain. The Gram-negative envelope forms a crucial barrier between the intracellular and harsh extracellular space and its maintenance is essential. The extracytoplasmic sigma factor, σ^E, is a major contributor to envelope homeostasis during exposure to stress-inducing conditions but much of its regulon remains poorly understood.

In this study, we aimed to understand the contribution of the heat shock sigma factor (σ^H)- and σ^E-regulated small heat shock proteins (sHsps) IbpA, IbpB and AgsA and the σ^E-regulated putative stress response protein STM1250 to the *Salmonella* stress response. Due to shared regulation and sequence homologies, we hypothesised that functional overlap exists between these proteins and that they contribute to maintaining cell envelope homeostasis during stress.

We have identified novel roles for the sHsps and STM1250 in *Salmonella* survival against hydrogen peroxide-associated oxidative stress and the cationic antimicrobial peptide polymyxin B. Furthermore, we demonstrated that a quadruple deletion mutant is significantly attenuated in the intramacrophage environment. In contrast, single gene mutants were not significantly attenuated or sensitive, indicating that functional redundancy exists between these proteins. In addition, this study is the first to address the function of STM1250, which was shown to be required for a functional *Salmonella* acid tolerance response.

Considering their functional redundancy, we sought to investigate whether the proteins of interest may co-operate through protein-protein interactions. Initial bacterial two-hybrid results identified novel heterogenous interactions between the sHsps and STM1250.

Put together, we have demonstrated that *Salmonella* sHsps can protect against stresses beyond heat shock. Moreover, this study highlights that investigating functional redundancy between stress responsive proteins has the potential to identify those that are important to pathogenesis and may aid in identifying much needed new therapeutic targets.

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List of Abbreviations

ABC	ATP-binding cassette
Amp	Ampicillin
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASP	Acid shock protein
ATP	Adenosine triphosphate
ATR	Acid tolerance response
AUC	Analytical ultracentrifugation
BAM	β-barrel assembly machinery
BTH	Bacterial two-hybrid
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
CDC	Centre for Disease Control and Prevention
cDNA	Complementary DNA
CFU	Colony forming unit
CGD	Chronic granulomatous disease
ChIP	Chromatin immunoprecipitation
Cm	Chloramphenicol
CO_2	Carbon dioxide
CPS	Capsular polysaccharide
CRP	cAMP receptor protein
Ct	Calculated threshold
$CuCl_2$	Copper chloride
$CuSO_4$	Copper sulphate
CV	Column volume
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EMSA	Electrophoretic mobility shift assay
ESR	Envelope stress response
FBS	Foetal bovine serum
FPLC	Fast protein liquid chromatography
FRT	Flippase recognition target
h	Hour
H_2O_2	Hydrogen peroxide

HRP	Horseradish peroxidase
IFN-γ	Interferon gamma
IL	Interleukin
IM	Inner membrane
iNOS	Nitric oxide synthase
iNTS	Invasive non-typhoidal *Salmonella*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
kDa	Kilodalton
L	Litre
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MCS	Multiple cloning site
MDa	Megadalton
MDH	Malate dehydrogenase
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	No-carbon essential
NEAA	Non-essential amino acids
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NLR	Nod-like receptor
NO	Nitric oxide
nt	Nucleotide
NTS	Nontyphoidal *Salmonella*
O_2	Molecular oxygen
O_2^-	Superoxide
OD	Optical density
OH^-	Hydroxyl radicals
O/N	Overnight
OM	Outer membrane
OMP	Outer membrane protein
$ONOO^-$	Peroxynitrite
ONPG	Ortho-nitrophenyl-β-galactoside
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PP	Peyer's Patch
PPIase	Peptidyl-prolyl cis/trans isomerase
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RBS	Ribosome binding site
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNAT	RNA thermometer
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROSE	Repression of heat shock gene expression
rpm	Revolutions per minute
SCV	*Salmonella* containing vesicle
SD	Standard deviation
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Standard error of the mean
sp	Species
SPI	*Salmonella* pathogenicity island
spp	Species (plural)
SR	SRP receptor
SRP	Signal recognition particle
STM	*Salmonella* Typhimurium
T3SS	Type III secretion system
Tat	Twin-arginine translocation
TBE	Tris/Borate/EDTA
TBST	Tris-buffered saline Tween
TCS	Two-component system
TLR	Toll-like receptor
Tm	Melting temperature
TMDH	Transposon-mediated differential hybridisation
TNF-α	Tumour necrosis factor-alpha
TraDIS	Transposon-directed insertion-site sequencing
TraSH	Transposon site hybridization
TSB	Tryptic soy broth
UDP	Uridine-diphosphate-galactose
uOMP	Unfolded outer membrane protein
UTR	Untranslated region

UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
WKL	White-Kauffmann-Le Minor
WT	Wild-type
XDR	Extremely drug resistant
µL	Microlitre
µM	Micromolar

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Chapter 1 Introduction

Work in this chapter contributed to the following article:

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1.1 *Salmonella* nomenclature and serovars

Salmonella is a Gram-negative bacterium and member of the Enterobacteriaceae family. The *Salmonella* genus is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *S. bongori* is rarely responsible for causing disease in humans, and is mostly found in the environment and cold-blooded species (Brenner et al., 2000). In contrast, *S. enterica* is divided into six subspecies (subsp.); *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI) and of these, only subtype I (*enterica*) is responsible for human disease. *Salmonella enterica* subsp. *enterica* is further divided into serovars, according to the White-Kauffmann-Le Minor (WKL) classification scheme, based on the presence of O-, H- and K- antigens present on the cell surface (Issenhuth-Jeanjean et al., 2014). These serovars are typically grouped (Figure 1) into those which cause typhoidal disease (*S.* Typhi and *S.* Paratyphi) and those which lead to a non-typhoidal disease type (common examples include *S.* Typhimurium, *S.* Enteritidis, *S*. Dublin, *S.* Choleraesuis, and *S.* Heidelberg).

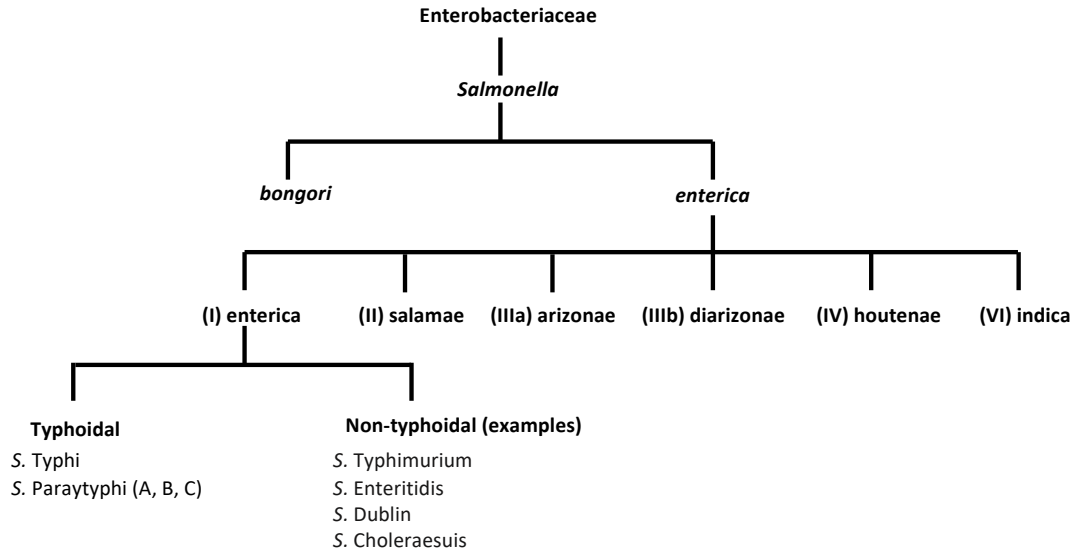


Figure 1. Nomenclature and serovar distribution of the *Salmonella* genus in the Enterobacteriaceae family. The *Salmonella* genus is divided into two species, *S. bongori* and *S. enterica*. The latter is further divided into six subspecies, of which subspecies I (*enterica*) is grouped into typhoidal and non-typhoidal serovars (common examples of each are shown).

According to the most recent WKL classification (Issenhuth-Jeanjean et al., 2014), approximately 2600 *Salmonella enterica* serovars have been identified to date. Generally, non-typhoidal *Salmonella* (NTS) serovars have a broad host range and are capable of infecting humans and a range of animals. In contrast, typhoidal serovars are defined as host-restricted and are only capable of causing disease in human hosts (Gordon et al., 2010).

1.2 *Salmonella* pathogenesis

Salmonella serovars are major causes of global morbidity and mortality with disease manifestations, depending on the serovar, including gastroenteritis (often referred to as salmonellosis), bacteraemia, enteric fever and a chronic carrier state (Coburn et al., 2007). Worldwide estimates state that *Salmonella* spp. (both non-typhoidal and typhoidal) are responsible for over 1.3 billion cases of disease annually (Coburn et al., 2007). In reality these figures are predicted to be even higher due to a lack of reporting, diagnosis and surveillance in developing areas, where *Salmonella* prevalence is at its highest (Amicizia et al., 2017, Pitzer et al., 2019). NTS are estimated to account for a greater proportion of *Salmonella* disease; a 2010 estimate reported that 93 million NTS cases and 155,000 deaths occurred annually (Majowicz et al., 2010). Typhoid fever cases in developing countries are high due to poor water sanitation and cases outside of the endemic regions of south-east Asia and sub-Saharan Africa are relatively rare. In fact, in 2014 it was reported that 99% of *S*. Typhi cases in the UK were related to recent travel to endemic areas (Public Health England, 2018).

1.2.1 Sources of infection

Zoonotic infections, those which are capable of transmission between animals and humans, are a primary cause of global enteric and systemic disease (Cantas and Suer, 2014, Asante et al., 2019). *Salmonella* serovars are capable of colonising a multitude of animals, with those used as livestock, including poultry, cattle and swine, of particular cause for concern when it comes to zoonotic infections (Chlebicz and Śliżewska, 2018). The intestinal tract of food-producing livestock is the primary reservoir for NTS serovars, with river water also proving to be a significant source of contamination (Liu et al., 2018). In comparison, *S.* Typhi and *S.* Paratyphi are human host-adapted and are unable to colonise animals (Eng et al., 2015, Demirbilek, 2017). *Salmonella* infection is generally transmitted via contaminated food and water; in 2010 it was estimated that 350 million cases of *Salmonella* infection arose through

the consumption of contaminated food products (Chlebicz and Śliżewska, 2018). Cases of food-/water-borne *Salmonella* disease are predicted to be higher than other enteric pathogens, with 52% of NTS and 37% of typhoidal serovar infections arising from this route (Besser, 2018). In a 2018 European Union review of 36 countries, salmonellosis was the second most common zoonotic disease, behind only campylobacteriosis (caused by *Campylobacter jejuni*) (European Food Safety Authority (EFSA), 2019).

Poor sanitation, extended animal-human interactions and exposure to environments in which *Salmonella* shedding has occurred are among the greatest risk factors for a *Salmonella* zoonotic infection (Rukambile et al., 2019). *Salmonella* outbreaks have been associated with the consumption of numerous foods, with the most common being raw/undercooked meats and eggs. However, non-animal foodstuffs should not be overlooked; notable *Salmonella* outbreaks have also occurred following consumption of salads (Berger et al., 2009), peanut butter (Sheth et al., 2011) and chocolate (Gill et al., 1983).

1.2.2 Gastroenteritis

As indicated above, a large majority of *Salmonella* cases are caused by the NTS serovars. In humans, infection with these serovars results in a localised gastroenteritis, commonly referred to as salmonellosis. Serovars capable of causing gastroenteritis in humans are host-generalists, with reservoirs in different animals. Despite the fact that these generalist serovars are genetically very similar, a 10-year study by Jones et al. (2008) across U.S. FoodNet states identified that disease severity can depend on the serovar of infection. Interestingly, *S.* Dublin serovars presented the highest fatality rate (3.0%) compared to *S.* Typhimurium (0.6%), while *S.* Dublin (64%) and *S.* Choleraesuis (57%) were significantly more likely to cause invasive bacteraemia compared to *S.* Typhimurium (6%) and *S.* Enteritidis (7%) (Jones et al., 2008).

Generally, salmonellosis symptoms arise between 6- and 72-hours post-inoculation and include vomiting, intestinal inflammation, abdominal cramps and diarrhoea. Salmonellosis is usually a self-limiting disease with the host immune system capable of clearing the infection within 4-7 days, without the need for therapeutic intervention. However, it is still estimated that 3 million deaths occur annually as a result of non-typhoidal *Salmonella* infection (Coburn et al., 2007).

1.2.2.1 Bacteraemia

NTS bacteraemia (bloodstream infection) is very rare with an estimated 5% of patients with salmonellosis developing the complication (Acheson and Hohmann, 2001). At risk groups include young children, especially babies and immunocompromised patients. As highlighted above, the occurrence of this form of the disease can be seen more commonly in infections arising from *S.* Choleraesuis and *S.* Dublin serovars (Chiu et al., 2004, Crum-Cianflone, 2008, Jones et al., 2008). Unlike *S.* Typhimurium and *S.* Enteritidis, these serovars have been shown to invade the bloodstream soon after the infection has begun. Endemic areas of *Salmonella* bacteraemia have been identified, with *S.* Choleraesuis a particular cause for concern in Taiwan, where an isolate with the highest rate of invasiveness has been identified (Chiu et al., 2004, Jean et al., 2006). Treatment of *Salmonella* bacteraemia is via antibiotics, typically ampicillin, quinolones or third-generation cephalosporins, although increasing incidences of antibiotic resistant infections mean that treatment options are increasingly limited (Acheson and Hohmann, 2001).

1.2.3 Enteric fever (typhoid and paratyphoid)

Salmonella enteric fever disease manifestations, namely typhoid and paratyphoid fever, are caused by the human host-restricted serovars *S.* Typhi and *S.* Paratyphi (A, B and C), respectively. In 2017, a global study estimated that there were 14.3 million cases of typhoid and paratyphoid disease, with *S.* Typhi causing 76.3% of these cases (Stanaway et al., 2019). Enteric fever is endemic in south and southeast Asia and sub-Saharan Africa where water supply and sanitation are typically poor (Figure 2). Incidences elsewhere remain low, and most cases that arise are associated with travel to an endemic region (Dave and Sefton, 2015). Studies have identified that the burden of paratyphoid fever is at its highest in Asia, although a lack of worldwide paratyphoid-specific surveillance and the similarities with typhoid symptoms make it difficult to accurately estimate the number of cases (Amicizia et al., 2017).

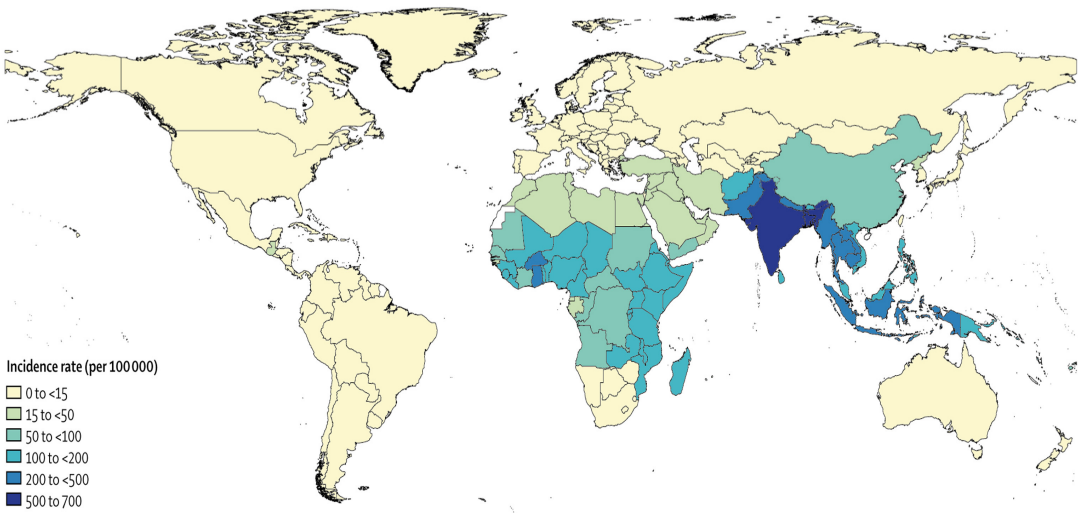


Figure 2. *Salmonella* enteric fever (typhoid and paratyphoid) incidences per 100,000 of the population in 2017. Countries are coloured based on the incidence rate per 100,000 people. Indicated by the dark blue colour, enteric fever incidences are high in south/south-east Asia (approximately 500 to 700 cases per 100,000) and sub-Saharan Africa (approximately 100 to 500 cases per 100,000) (Stanaway et al., 2019).

In contrast to salmonellosis, the incubation period of enteric fever, prior to the onset of symptoms, is typically up to 2 weeks following inoculation (Coburn et al., 2007). In general, symptoms begin with a mild fever, dull frontal headache, fatigue and myalgia (muscle pain). As the infection progresses, the fever rises and vomiting and diarrhoea can occur. These symptoms are relatively non-specific and can result in a lack of diagnoses, particularly in the endemic countries (Kumar and Kumar, 2017).

Treatment of enteric fever is essential and in serious cases the mortality rate can be as high as 30% without therapeutic intervention (Buckle et al., 2012). Advances in the understanding of the pathogenesis of enteric fever, in addition to the development of treatments and preventative measures, can be significantly attributed to the use of *S.* Typhimurium as a laboratory model of typhoid disease. Despite being a well-studied cause of salmonellosis, *S.* Typhimurium infects mice systemically and causes an enteric, typhoid-like disease - an invaluable resource for research (Santos et al., 2001).

1.2.3.1 Chronic carrier stage

Both *S.* Typhi and *S.* Paratyphi are capable of colonising the gall bladder and establishing a chronic carrier infection, a disease state that develops following recovery from the acute phase of infection (Gupta et al., 2006). Within the gall bladder niche, *Salmonellae* persist and successfully evade the host immune system (see

1.2.6) (Gonzalez-Escobedo and Gunn, 2013). It is estimated that approximately 2-5% of typhoid patients develop this stage of the disease (Gunn et al., 2014) and *Salmonella* can be shed in the stools for many years after infection (Gupta et al., 2006). Since *S.* Typhi and *S.* Paratyphi are human host-restricted, it is the chronic carrier patients that serve as key environmental reservoirs for these serovars (Gal-Mor, 2018). Patients in the chronic carrier stage are asymptomatic, which poses problems in terms of monitoring and controlling the spread of enteric disease (Gunn et al., 2014). Furthermore, *Salmonella* shedding from these asymptomatic patients has been an important source of infection in non-epidemic countries such as the United States (Gupta et al., 2006).

1.2.4 Invasive non-typhoidal *Salmonella*

Invasive non-typhoidal *Salmonella* (iNTS) is an emerging *Salmonella* infection, caused by serovars that are usually associated with salmonellosis. The disease is endemic in sub-Saharan Africa, where the *S.* Typhimurium strain ST313 is particularly prevalent (Kingsley et al., 2009, Feasey et al., 2012, Parsons et al., 2013, Canals et al., 2019, Gilchrist and MacLennan, 2019). Current global estimates have suggested that there are over 3 million cases of iNTS resulting in at least 600,000 deaths annually, but accurate statistics are difficult to determine, with a distinct lack of microbiological diagnostic facilities in the endemic regions (Balasubramanian et al., 2019). Typical salmonellosis symptoms are often absent and are instead replaced by fever, respiratory symptoms and hepatosplenomegaly (enlargement of the liver and spleen). HIV is a key risk factor, in both adults and children, for the contraction of iNTS disease. An HIV infection is thought to provide an immunological niche for iNTS colonisation (Feasey et al., 2012, Okoro et al., 2012) and it is the immunological defects within the host that enable the bacterial colonisation; specifically dysregulated cytokine production enabling *Salmonella* persistence, impaired serum killing and dysregulation of epithelial barrier maintenance caused by the loss of CD4+ T-cells in the gut mucosa (Feasey et al., 2012). Initial reports suggested that ST313 was human host-restricted; however, this was disproved by Parsons et al. (2013) who demonstrated that ST313 causes severe systemic infection in chickens with rapid colonisation of the liver and spleen following inoculation.

More recently, a novel iNTS isolate (ST34) was identified in Vietnam, the first to have been identified outside of sub-Saharan Africa (Mather et al., 2018), emphasising the importance of continued global surveillance of this emerging disease-type.

1.2.5 *Salmonella* molecular mechanisms of infection

1.2.5.1 *Salmonella* cell tropisms and routes of infection

Following ingestion and passage through the highly acidic environment of the stomach (see Chapter 4), *Salmonella* crosses the epithelial barrier within the small intestine where it is capable of invading and colonising numerous cell types. *Salmonella* crosses the intestinal epithelium by the preferential invasion of M-cells in Peyer's patches (PP) before phagocytosis by macrophages, neutrophils or dendritic cells (Jones et al., 1994, Santos and Bäumler, 2004). Although *Salmonella* exhibits a tropism for M-cells, with an increased rate of adhesion and invasion of this cell type, during bovine infection *S.* Typhimurium has also been detected in intestinal goblet cells and enterocytes (Santos and Bäumler, 2004). Non-invasive NTS serovars cause a self-limiting gastroenteritis infection which remains localised in the intestine (1.2.2). In contrast, during a systemic infection, *Salmonella* are released into the bloodstream and continue to colonise distal sites including the liver, spleen and bone marrow (Everest et al., 2001). Following replication at these sites, secondary bacteraemia occurs and bacteria subsequently colonise the gall bladder, from which re-infection of the small intestine is possible (Everest et al., 2001). During this secondary exposure of the small intestine, inflammation, ulceration and ultimately necrosis of the PP occurs. In serious cases (typically ~5% of patients), PP perforations can lead to septicaemia, with a mortality rate of up to 83% depending on how quickly treatment can be administered (Everest et al., 2001).

1.2.5.2 *Salmonella* virulence factors and pathogenicity islands

Virulence factors are central to the ability of a pathogen to survive and replicate within a host (Cross, 2008). *Salmonella* virulence factors include adhesins and fimbriae, flagella, virulence plasmids and type III secretion systems (T3SS), with their associated effector proteins (Dos Santos et al., 2019). A large number of virulence factors are encoded on *Salmonella* pathogenicity islands (SPIs), formed of gene clusters, most of which have been acquired over the course of *Salmonella* evolution by horizontal gene transfer (Haneda et al., 2009).

There are at least 17 SPIs identified in the *Salmonella* genome, although they are not completely conserved across all serovars of the species (Ilyas et al., 2017). There are 11 SPIs conserved between *S.* Typhimurium and *S.* Typhi (SPI- 1 to 6, 9, 11, 12, 13 and 16), while SPI-14 is specific to *S.* Typhimurium and SPI-7, 15, 17 and 18 are specific to *S*. Typhi (Sabbagh et al., 2010). SPIs are typically characterised by their low percentage GC content (37-47% for SPIs compared with the *Salmonella*

Typhimurium genome average GC content of 52.2% (Papanikolaou et al., 2009)), the inclusion of genes associated with virulence properties of *Salmonella* and their proximity to/inclusion of mobile genetic elements such as tRNA loci, indicating their acquisition via horizontal transfer (Silva et al., 2012). While both SPI-1 and SPI-2 are likely to have been acquired via a single horizontal transfer event, following divergence from the *Escherichia coli* common ancestor (Lerminiaux et al., 2020) other SPIs such as SPI-3 and SPI-5 have mosaic structures, where not all genes in the island are functionally similar (Blanc-Potard et al., 1999). These mosaic islands are understood to have evolved through multiple horizontal transfer events (Blanc-Potard et al., 1999, Ilyas et al., 2017). The SPIs are not conserved in closely related enterobacterial species including *E. coli*, *Yersinia pestis* and *Shigella flexneri*, therefore acquisition of these genomic regions is understood to have occurred over 100 million years ago, following divergence of *Salmonella* and closely related species from a common ancestor (Hacker et al., 1997).

1.2.5.2.1 SPI-1 and SPI-2

The contribution of each known SPI to *Salmonella* pathogenicity is not fully understood and each SPI has not been studied to the same extent. SPI-1 and SPI-2 are to date the most well-characterised, both of which encode a T3SS responsible for injecting effector proteins into the host cell cytosol (for recent reviews see Lou et al. (2019) and Jennings et al. (2017)). Each of the two T3SS are expressed at different stages of the infection process, with SPI-1 responsible for invasion of intestinal epithelial cells and SPI-2 required for survival in the *Salmonella* containing vacuole (SCV). The effector proteins secreted by each system are involved in the modification of host cell processes. In general, these modifications promote bacterial uptake and increase intracellular survival.

With an average GC content of 42%, SPI-1 is a 40 kb region encoding 39 genes required for the formation of a T3SS (T3SS-1) and for the associated effector proteins and is required for host cell invasion (Lou et al., 2019). The acquisition of SPI-1 is seen as pivotal in the evolution of *Salmonella* as a pathogen with the ability to cross the gut epithelium (Bäumler, 1997). It is predicted that SPI-1 was acquired at the point of divergence of the *Escherichia* and *Salmonella* genera because SPI-1 is conserved among all *Salmonella* species and serovars, including the non-pathogenic *S. bongori* (Bäumler et al., 1998). Encoded within the SPI-1 region are multiple proteins required for the formation of the needle and syringe complex of the T3SS-1. The system is formed of a basal inner membrane ring (PrgH/K and InvG/H), a needle structure (PrgI and PrgJ) protruding from the outer membrane (OM) and into the extracellular space

and a translocon (SipB, C and D) which sits within the host cell membrane and through which additional effector proteins are secreted (Collazo and Galán, 1997, Kubori et al., 1998, Kubori et al., 2000, Kimbrough and Miller, 2000, Kimbrough and Miller, 2002, Deng et al., 2017).

There are at least 20 effector proteins that are secreted through the T3SS-1 complex (Boonyom et al., 2010), including AvrA, SipA, SipB, SipC, SipD, SopB, SopE and SopE2. While the SipA, B, C and D effector proteins are encoded within SPI-1, additional effector proteins are located in other pathogenicity islands or mobile genetic elements (Galán, 2001) – for example, SopB is located on SPI-5 (Dos Santos et al., 2019). Translocation of effector proteins results in host cell membrane ruffling and remodelling of the actin cytoskeleton, both of which promote bacterial uptake by the epithelial cell (Malik-Kale et al., 2011, Srikanth et al., 2011).

Also comprised of a 40 kb region, SPI-2 encodes at least 40 genes and is formed of two segments, that are predicted to have been acquired in separate horizontal transfer events (Marcus et al., 2000). It is the general consensus that the smaller of the two segments was acquired first, due to sequence similarity with *S.* Bongori (Hensel et al., 1999b, Marcus et al., 2000). This segment encodes genes for tetrathionate metabolism (*ttrRS*, *ttrBCA*) and is not required for systemic murine infection (Hensel et al., 1999a, Hansen-Wester et al., 2004). The larger segment is only located in *S. enterica* species and is required for systemic infection (Hensel et al., 1999b, Marcus et al., 2000). Genes in this segment are broadly divided into four functional operons; *ssa* (T3SS-2 components), *ssr* (regulation of T3SS-2 expression), *ssc* (T3SS-2 chaperones) and *sse* (T3SS-2 effectors) (Marcus et al., 2000). In a similar manner to the SPI-1 encoded T3SS-1, the key components of the T3SS-2 are a basal inner membrane ring (SsaV), a needle (SsaG) protruding from the OM (via SsaC) and a translocon for the delivery of effector proteins into the host cell (SseB, C and D) (Yu et al., 2018). There are at least 28 effector proteins currently known to be secreted by SPI-2, but the precise proteins secreted can differ between *Salmonella* serovars (Jennings et al., 2017). Conserved effector proteins include SseF/G, PipB, SteA, SifA, SteD and PipB2 while intestinal serovars secrete effectors (e.g. SseL, SifB, SlrP, SopD2) that are not encoded by extra-intestinal/systemic serovars (Jennings et al., 2017). Overall, SPI-2 encoded effector proteins are involved in the maintenance of the integrity of the SCV, through the formation of *Salmonella*-induced filaments (Sif) (reviewed in detail in (Knuff and Finlay, 2017)), host cytoskeleton modulation and disruption of immune signalling cascades (Ruiz-Albert et al., 2002, Srikanth et al., 2011, Figueira and Holden, 2012).

Expression of SPI-2 is induced by environmental factors associated with the intracellular environment including acidic pH, low magnesium (Mg^{2+}) and low calcium (Ca^{2+}). Three two component systems (TCS) contribute to SPI-2 regulation: SsrAB, (SPI-2 encoded), PhoPQ (1.5.2.2) and OmpR/EnvZ (4.1.1.1.4)

1.2.6 Immune responses against *Salmonella* infection

1.2.6.1 Innate Immunity

The innate immune system is a non-specific response which forms a major part of the barrier to infection and is involved in the recognition of foreign invaders. The response can be broadly divided into two components: inflammation and phagocytosis. When pathogens such as *Salmonella* invade the intestinal mucosa, inflammation is induced which, in turn, increases blood flow to the area and recruits immune cells and cytokine release (Broz et al., 2012).

Pattern recognition receptors (PRRs) form a crucial part of the defence against pathogens and in the host's recognition of infection. PRRs include toll-like receptors (TLRs) and nod-like receptors (NLRs) and they both play a central role in the detection of immunogenic pathogen associated molecular patterns (PAMPs), alerting the immune system that an invading bacterium is present (Kawai and Akira, 2009). In humans there are ten members of the TLR family, of which six (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) are located on the cell surface and four (TLR 3, TLR7, TLR8, TLR9) are located in the endosome (Kawasaki and Kawai, 2014).

Salmonella pathogen associated molecular patterns include lipopolysaccharide (LPS), flagella, CpG regions of DNA and curli amyloid fibres located in the *Salmonella* biofilm matrix (Broz et al., 2012, Wang et al., 2020). Generally each TLR binds to a specific PAMP, for example TLR5 and TLR11 bind to bacterial flagellin, TLR4 recognises bacterial LPS and TLR9 recognises CpG regions of DNA (Broz et al., 2012). TLR2 is an exception to the rule and, in co-operation with TLR1 or TLR6, can detect the presence of multiple PAMPs including lipoproteins and peptidoglycan (Broz et al., 2012, Kawasaki and Kawai, 2014). Ultimately, the activation of PRRs, via recognition of PAMPs, leads to the induction of pro-inflammatory cytokines, including interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), and interleukins 1 and 6 (IL-1 and IL-6), with the purpose of amplifying the immune response (Mogensen, 2009). In comparison to TLRs, the purpose of NLRs is to detect intracellular PAMPs – they are therefore highly important in the detection of intracellular bacteria such as *Salmonella* (Kanneganti et al., 2007). Among the most well characterised NLRs are NOD1 and NOD2 which, following PAMP detection, form

a dimer and activate NF-κB dependent pro-inflammatory cytokine production. Other NLRs include those which induce the formation of the inflammasome, a macromolecular signalling complex that activates Caspase-1, leading to production of IL-18 and IL-1B (Martinon et al., 2002, Petrilli et al., 2005). In addition, inflammasome-dependent Caspase-1 activation induces pyroptosis, an inflammatory driven form of programmed cell death first identified during *Salmonella* and *Shigella* infections (Fink and Cookson, 2005). In comparison to apoptosis, in which cell death is described as 'silent' because cell contents are not typically released, during pyroptosis the cell contents – inclusive of cytokines, PAMPs and the invading pathogen – are released and can be detected by neighbouring immune cells (Sellin et al., 2014, Knodler et al., 2014).

In all cases described above, pro-inflammatory cytokine production enables further recruitment and activation of phagocytes including macrophages, neutrophils and dendritic cells. Neutrophils are a primary line of defence and their deficiency, as seen in HIV patients with neutropenia, commonly leads to the development of bacteraemia (Pham and McSorley, 2015). Macrophages play a crucial role in the elimination of *S.* Typhimurium due to their expression of natural resistance-associated macrophage protein 1 (Nramp1), also called Slc11a1 (Govoni and Gros, 1998). Although Nramp1 has been shown to limit intracellular survival of pathogens, its precise mechanisms remain unclear. Despite this, studies have suggested that Nramp1 prevents complete maturation of the *Salmonella* containing vacuole via export of metal ions (in particular manganese, cobalt and iron), thereby limiting *Salmonella* proliferation within the intracellular environment (Cuellar-Mata et al., 2002, Cunrath and Bumann, 2019). In addition to Nramp1, macrophages also express the inducible nitric oxide synthase (iNOS) and phagocyte oxidase (phox) for production of toxic nitric oxide (NO) and reactive oxygen species (ROS), respectively (see Chapter 4 for further detail) (Vazquez-Torres and Fang, 2001a).

Dendritic cells (DCs) recognise *Salmonella* LPS and flagellin, and as they mature they increase antigen presentation and migrate to the T-cell area of lymphoid tissues, presenting antigens to naïve T-cells to initiate the adaptive immune system (Théry and Amigorena, 2001, Griffin and McSorley, 2011, Pham and McSorley, 2015). In its role as an intracellular pathogen, *Salmonella* can induce apoptosis of DCs, rendering them unable to function as an antigen presenting cell (APC). However, at the same time, apoptosis releases antigenic material which can be phagocytosed by nearby DCs and this instead can be presented to T-cells (Wick, 2003). Although the presence of LPS enables recognition of *Salmonella* by DCs, the O-antigen is important for the

ability of *Salmonella* to survive within these cells. Surprisingly, *Salmonellae* deficient in the O-antigen component of LPS have increased uptake by DCs and reduced intracellular survival (Wick, 2003).

1.2.6.2 Adaptive immunity

While the innate immune response has been shown to be sufficient in the prevention of the progression of a *Salmonella* infection, the contribution of the adaptive (humoral) arm of the immune system remains less well-understood. *Salmonella* immune evasion capabilities are evident in its ability to suppress B-cell production (lymphopoiesis) and B-cell activation (Takaya et al., 2020). In addition, a recent study reported that *Salmonella* prevents immune 'memory' by suppressing IgG production via reduction of IgG-secreting plasma cells in the bone marrow, in a SPI-4 dependent manner (Männe et al., 2019). As described below, vaccination for *Salmonella* is currently only available for the Typhi serovars, but human vaccination against NTS is not possible. This is largely due to the fact that there are a vast number of different NTS serovars, increasing the difficulty of identifying an appropriate vaccine that can target this variety. However, on a molecular level, Takaya et al. (2020) highlighted that the SPI-4 encoded *siiE* gene is a pseudogene in *S.* Typhi; therefore, this serovar lacks this specific mechanism of immune memory suppression. It has been suggested that a lack of *siiE* in *S.* Typhi may have contributed to the development of an effective vaccination, which has not yet been achieved for NTS (Takaya et al., 2020).

1.3 *Salmonella* prevention and treatment

As a pathogen of global importance and significance, the management of *Salmonella* disease through treatment and prevention is highly important. *Salmonella* sp. are a high priority pathogen for research, according to the World Health Organisation (WHO), providing evidence that continued surveillance and new treatment mechanisms are surely required (Tacconelli et al., 2018). Current antibiotics are not expected to stand the test of time with multi-drug resistant strains continually emerging. Preventative vaccines are available in the clinic, but with downfalls, and *Salmonella* infections continue to affect millions of people worldwide.

1.3.1 Antibiotics

In otherwise healthy individuals, infection with non-typhoidal *Salmonella* serovars rarely requires therapeutic intervention. For immunocompromised individuals, the elderly and young children, antibiotics may be required to clear the infection.

Conversely, infection with typhoidal serovars requires antibiotic treatment. Chloramphenicol was identified as an effective treatment for typhoid fever in 1948 and was used with a high rate of success for about 40 years (Butler et al., 1999, Levine and Simon, 2018). However, as is the ever increasing trend with continued antibiotic use, chloramphenicol resistant *S.* Typhi soon emerged. These resistant isolates were first identified in the early 1970s, with a large epidemic in Mexico City in 1972 (Olarte and Galindo, 1973), and further outbreaks associated with chloramphenicol resistant *S.* Typhi strains were reported throughout the following decades (Levine and Simon, 2018). Furthermore, the emergence of multi-drug resistant (MDR) strains, which had acquired R- (resistance) factor plasmids, added a further cause for concern; these strains led to a reduced efficacy of many other first line antibiotics including ampicillin, trimethoprim, sulphonamides, streptomycin and tetracycline (Coovadia et al., 1992, Kalra et al., 2003, Levine and Simon, 2018). By the 1990s, ciprofloxacin, ofloxacin and fluoroquinolone drugs replaced chloramphenicol as the first choice antibiotic for typhoid treatment (Levine and Simon, 2018). Currently, the WHO still recommends use of the antibiotics listed above (where possible), in addition to fluoroquinolone antibiotics, for the treatment of typhoid fever (Browne et al., 2020). The increase in global antibiotic resistance is well documented and this is certainly a cause for concern for treatment of *Salmonella* infection. Surveillance of such cases is essential for infection control and in the U.S., the Centre for Disease Prevention and Control (CDC) recently reported the occurrence of 212,500 drug-resistant nontyphoidal *Salmonella* infections and 4,100 drug resistant *S.* Typhi infections (CDC, 2019). Highlighting the need for new treatment options, in a recently published priority pathogens list the WHO indicated that fluoroquinolone resistant *Salmonella* sp. are within a “high priority” group of pathogens, where researchers are recommended to focus efforts to identify novel therapeutics (Tacconelli et al., 2018).

1.3.2 Vaccinations

The first *Salmonella* vaccine was in the form of an inactivated whole cell vaccination and was brought into use in 1896. Unfortunately, due to its high reactogenicity (a high number of side effects), including fever, headaches and severe localised pain, the vaccination was not introduced into the clinic for long-term public use (Guzman et al., 2006, MacLennan et al., 2014). In the present day, there are two vaccinations available in the clinic for prevention of *S.* Typhi and the resulting typhoid fever: the Vi capsular polysaccharide (Vi CPS) and the Ty21a vaccination.

1.3.2.1 Vi capsular polysaccharide

The Vi CPS is a subunit based vaccination, derived from the *S.* Typhi Ty2 strain. The Vi CPS is a key *S.* Typhi virulence factor, encoded by the 1 kb *viaB* locus located on the SPI-7 region of the genome (Wilson et al., 2011). Vi CPS enables immune evasion and is expressed once *S.* Typhi has transitioned into the ileal mucosa of the host (Tran et al., 2010). The current Vi CPS vaccine induces a protective response to *S.* Typhi infection only. However, a recent study identified that recombinant production of *S.* Typhi Vi CPS in the *S.* Paratyphi A strain conferred protection against both of these serovars in the form of a live vaccine (Xiong et al., 2017). Incidences of paratyphoid infections are consistently increasing, therefore developments such as this are crucial to advancing the treatment and prevention of multiple invasive enteric diseases.

1.3.2.2 Ty21a

In contrast to the Vi CPS, Ty21a (Vivotif®) is a live attenuated vaccine, taken in capsular form, containing a whole-cell mutated form of the *S.* Typhi Ty2 strain, which already carries a mutation in the *rpoS* gene, encoding the general stress response sigma factor (Robbe-Saule and Norel, 1999) (see 1.5.1.1 for more detail on RpoS).

The Ty21a mutant lacks the *galE* gene, encoding uridine-diphosphate-galactose (UDP-gal)-4-epimerase, rendering it unable to convert UDP-glucose to UDP-galactose and vice versa (Germanier and Fiirer, 1975). UDP-galactose is an important component of the LPS O-antigen and without the LPS PAMP, *Salmonella* are avirulent (Amicizia et al., 2017). During production of the live vaccine strain Ty21a, *Salmonella* is supplemented with galactose and once *in vivo*, exogenous sources of galactose are also imported by *Salmonella* (Levine, 2018). This results in the partial formation of LPS on the cell surface. The key factor that renders the Ty21a strain avirulent is the fact that the mutated form of *Salmonella* is unable to metabolise the imported exogenous galactose; galactose accumulates within the cytoplasm in the form of UDP-galactose and galactose-1-phosphate ultimately leading to lysis and cell death (Germanier and Fiirer, 1975). Therefore, thanks to the partial formation of an immunogenic LPS but the ultimate lysis due to galactose accumulation, the bacteria themselves are avirulent but still able to elicit an immune response as a vaccine strain (Germanier and Fiirer, 1975).

Although comprised of an *S.* Typhi strain, current evidence suggests that the Ty21a vaccine may also provide cross protection to *S.* Paratyphi A and B, although further

studies are required to determine the efficacy of this protection (Pakkanen et al., 2012).

1.3.2.3 Future directions for vaccine development

Key problems exist with the current Vi CPS and Ty21a vaccinations. Firstly, it is recommended that booster vaccinations for both versions are given every 2-3 years and as a result this is not seen as a sustainable method of prevention, especially in the developing countries in which typhoid fever is endemic. Moreover, current vaccines can prevent acute infections but do not protect patients in the chronic carrier stage (Gupta et al., 2006). Secondly, both the Ty21a and Vi vaccination are not recommended for young children, one of the most susceptible demographic groups. Interestingly, conjugate vaccinations are recently emerging including a *Salmonella* conjugate vaccine (comprised of the *Salmonella* Vi CPS antigen and a detoxified variant of the tetanus toxoid) which was shown to be effective in children <2 years (Mitra et al., 2016). To date, there are three *Salmonella* tetanus toxoid vaccines (Typbar-TCV (Bharat Biotech), PedaTyph (BioMed) and Vi-TT (Zydus Cadila)) used in the clinic, but currently these vaccines are only licensed for use in India (Sahastrabuddhe and Saluja, 2019).

Finally, current licensed vaccinations only protect against typhoid fever caused by *S.* Typhi, no known protection is provided against Paratyphi, iNTS or NTS serovars. Although *S.* Typhi is responsible for the majority of enteric fever cases, the lack of a paratyphoid vaccination is still a cause for concern (Dave and Sefton, 2015). In addition, although iNTS and NTS serovars are usually self-limiting, serious cases and mortality do occur. As a result, research continues to try and identify new, longer-lasting and cross-protective *Salmonella* vaccine candidates that can be used as more effective methods of disease prevention.

1.4 The Gram-negative envelope

Forming a crucial protective barrier between the intracellular and harsh extracellular environment, the Gram-negative bacterial envelope is essential for cell viability and is therefore an attractive target for novel therapeutic intervention. The cell envelope is formed of an inner and outer membrane, which are separated by an aqueous periplasmic space containing a thin layer of peptidoglycan (Figure 3).

The inner membrane (IM) is comprised of a phospholipid bilayer and contains two systems for protein secretion, Sec and Tat (1.4.1.1). In addition to protein secretion systems, the IM also consists of proteins involved in respiration and energy

production, lipid biosynthesis and protein translocation and transport (Silhavy et al., 2010). In comparison to the IM, the outer membrane (OM) is asymmetrical in structure with phospholipids on the inner leaflet and LPS located on the outer leaflet (Nikaido, 2003). Located within the OM are transmembrane β-barrel proteins which are uniquely found in bacterial, mitochondrial, and chloroplast outer membranes (Fairman et al., 2011). These proteins are often collectively referred to as outer membrane proteins (OMPs) and their roles include molecule transport, protein/LPS transport and insertion, signalling, adhesion and virulence (Rollauer et al., 2015).

Since the OM forms a crucial barrier between the cell and the environment, the biogenesis and transport of OMPs is a highly regulated and controlled process. This section will introduce the process of OM biogenesis and highlight the key mechanisms involved, before an introduction to the bacterial stress responses which are paramount in the maintenance of both cell envelope and cytoplasmic homeostasis (see 1.5).

1.4.1 Outer membrane biogenesis and the β-barrel assembly machinery

OM biogenesis has been extensively studied in *E. coli* and the process is well conserved among Gram-negative bacterial species. Further studies have been performed in recent years, extending the research into species such as *Salmonella enterica*, which have highlighted differences in the process for example different essentiality and roles in virulence of the β-barrel assembly machinery (BAM) complex components (1.4.1.3).

This section will describe the major stages of OM biogenesis and maintenance in addition to highlighting the key proteins involved.

1.4.1.1 OM protein transport

Proteins destined for the envelope must be transported across the inner membrane (IM). To this end, two key protein secretion systems are located within the IM – the general secretion pathway (Sec pathway) and the twin arginine translocation pathway (Tat-pathway). The majority of secreted proteins are transported via the Sec pathway, formed of the SecYEG IM channel and the SecA motor ATPase (Kuhn et al., 2017, Tsirigotaki et al., 2017, Smets et al., 2019). Proteins destined for this system encode an N-terminal signal peptide and are transported in their unfolded state (Collinson et al., 2015). Transport across the IM is an energy dependent process which requires SecA hydrolysis of ATP. Studies have also shown that transport is still possible in the

absence of SecYEG, but in these cases signal specificity is lost (Hsieh et al., 2011, Lin et al., 2012). In contrast to Sec, the Tat system, comprised of the TatA, TatB and TatC proteins in Gram-negative bacteria is required for the transport of folded proteins and those containing metal cofactors (Palmer and Berks, 2012).

From the periplasmic leaflet of the IM, uOMPs must be transported in their unfolded state to the BAM complex for folding and insertion into the OM. This role is primarily performed by a core group of periplasmic chaperones SurA, Skp and DegP (see 1.4.1.2). However, our understanding of OM biogenesis and the range of periplasmic proteins involved is continually expanding and the complete process is not fully characterised. With numerous components of the BAM complex and periplasmic protein transport machineries being associated with pathogenesis, an improved understanding of this complex process will certainly aid in the search for novel therapeutic agents.

1.4.1.2 The core OM biogenesis chaperones: SurA, Skp and HtrA (DegP)

The periplasmic space is devoid of ATP and contains numerous ATP-independent chaperones and proteases, important for maintenance of periplasmic homeostasis and for transport of OMPs from the IM transport machinery. A crucial feature of these molecular chaperones is their ability to recognise the exposed hydrophobic domains and residues of unfolded/misfolded proteins (Spiess et al., 1999).

SurA, Skp and HtrA (DegP) are three key periplasmic chaperones involved in OMP biogenesis (Sklar et al., 2007). Coupled to this primary role, these core periplasmic chaperones have also been reported to be involved in the virulence of bacterial pathogens including *Salmonella* and *E. coli* (see 1.4.3).

Survival protein A (SurA) is a 47 kDa parvulin-like peptidyl-prolyl cis/trans isomerase (PPIase) and molecular chaperone, first identified as an essential protein for stationary phase growth in *E. coli* (Tormo et al., 1990, Lazar et al., 1998). Parvulin PPIases assist in protein folding via catalysis of cis/trans isomerization of peptidyl bonds around proline residues, which without catalysis is a rate limiting step in protein folding (Jakob et al., 2009). Crystallographic studies revealed that SurA has an “asymmetric dumbbell” conformation (Bitto and McKay, 2002). The protein is formed of an N-terminal region, two consecutive parvulin-like domains (I and II) and a C-terminal tail. Alone, domains I and II do not exhibit chaperone activity and only domain II exhibits PPIase activity (Behrens-Kneip, 2010). SurA functions primarily as a chaperone and transports uOMPs to the BAM complex via interactions with the major transmembrane component, BamA (Behrens et al., 2001, Sklar et al., 2007, Vuong

et al., 2008). SurA is crucial for maintenance and biogenesis of the OM and cell envelope; SurA has been localised at and can associate with the OM (Hennecke et al., 2005). *E. coli surA* mutants are highly sensitive to SDS/EDTA and rifampicin treatment and exhibit a constitutively active σ^E response, highlighting that deletion of *surA* severely disrupts periplasmic homeostasis (1.5.1.3) (Behrens et al., 2001). Various proteins have been identified as targets of SurA including the OM porin and *E. coli* bacteriophage lambda receptor LamB and the OMPs, OmpA, OmpC and OmpF (Rouvière and Gross, 1996).

Seventeen kilodalton protein (Skp) is encoded within the *bamA* (1.4.1.3) genomic region and functions in cooperation with SurA. Skp adopts a trimeric jellyfish-like shape with structural similarity to the prefoldin protein of Eukaryotes and Archaea (Walton and Sousa, 2004). *E. coli* and *Salmonella* Δ*skp*Δ*surA* double mutations are lethal and it is understood that the two proteins function on two separate pathways of OM biogenesis; in a *surA* mutant, the presence of Skp compensates for the loss of SurA and vice versa (Sklar et al., 2007). Unlike the Δ*surA* mutant, deletion of *skp* does not significantly compromise the integrity of the OM but Skp has been shown to play a role in the folding of specific OMPs, including OmpA, via direct interactions with BamA (Patel and Kleinschmidt, 2013).

HtrA (also known as DegP) is a periplasmic protein possessing both chaperone and protease function (Spiess et al., 1999). Originally described as a small heat shock protein (see 3.1.1), HtrA has also been shown to respond to heat, osmotic, pH and hydrogen peroxide (H_2O_2) induced cellular stress and is regulated by the *rpoE* sigma factor (1.5.1.3) and the CpxAR (1.5.2.1) envelope stress response systems. The chaperone and protease activities of HtrA are controlled in a temperature dependent manner. At low temperatures, HtrA predominantly functions as a chaperone and responds to the presence of misfolded proteins in the periplasm (Spiess et al., 1999). At elevated temperatures, the rate of protein damage and misfolding is increased and the protease activity of HtrA is prominent, degrading proteins that are beyond repair (Spiess et al., 1999, Lewis et al., 2009). HtrA is conserved across many Gram-negative bacteria. However, differences in its specific role differ between bacterial species; for example, HtrA is essential in *E. coli* for growth above 42 °C (Lipinska et al., 1989) but *Salmonella* Δ*htrA* mutants are viable at this temperature (Lewis et al., 2009). In a similar manner to the above Δ*surA*Δ*skp* mutant, double deletions of *surA* and *htrA* are lethal in *E. coli*, but in contrast, Δ*skp*Δ*htrA* mutants survive (Sklar et al., 2007). It is therefore understood that the primary pathway features SurA only while Skp and DegP provide an alternative pathway to either protect against the absence

of SurA or to support during stress when levels of misfolded and damaged proteins are extremely elevated (Sklar et al., 2007).

1.4.1.3 The β-barrel assembly machinery

The BAM complex is central to the process of uOMP folding and insertion into the OM. The complex is formed of the OM β-barrel protein BamA (formerly YaeT) and four accessory lipoproteins BamBCDE (formerly YfgL, NlpB, YfiO and SmpA, respectively). Each of the five genes encoding BAM complex components are regulated by the extracytoplasmic sigma factor σ^E (see 1.5.1.3), thereby directly linking their roles in OM assembly with maintenance of cell envelope/periplasmic homeostasis during stress.

The core component of the BAM complex, BamA, was first discovered in *Neisseria meningitidis* as the essential OMP, Omp85 (Voulhoux et al., 2003). Later studies have confirmed that BamA is essential in all Gram-negative bacteria and is required for correct function of the BAM complex (Tommassen, 2010, Misra, 2012). In contrast, the essentiality of the remaining accessory lipoproteins varies among bacterial species. In *E. coli,* BamD is also essential for correct BAM complex function but in contrast, *Salmonella bamD* mutants are viable (Fardini et al., 2009).

The BAM complex has been associated with virulence in multiple Gram-negative species including *Salmonella* and *Yersinia enterocolitica* and therefore poses as a potential novel therapeutic target (see 1.4.3). Although much of the BAM complex characterisation has been performed using *E. coli*, *Salmonella bamE* mutants have been reported to be attenuated during murine infection (Lewis et al., 2008). In addition, *S.* Enteritidis *bamB* and *bamD* mutants are less virulent and expression of flagella and T3SS are both reduced (Amy et al., 2004, Fardini et al., 2007, Fardini et al., 2009). *Y. enterocolitica bamB* mutants are also attenuated during murine infection, with significant reductions observed in bacterial load, particularly in the spleen (Weirich et al., 2017).

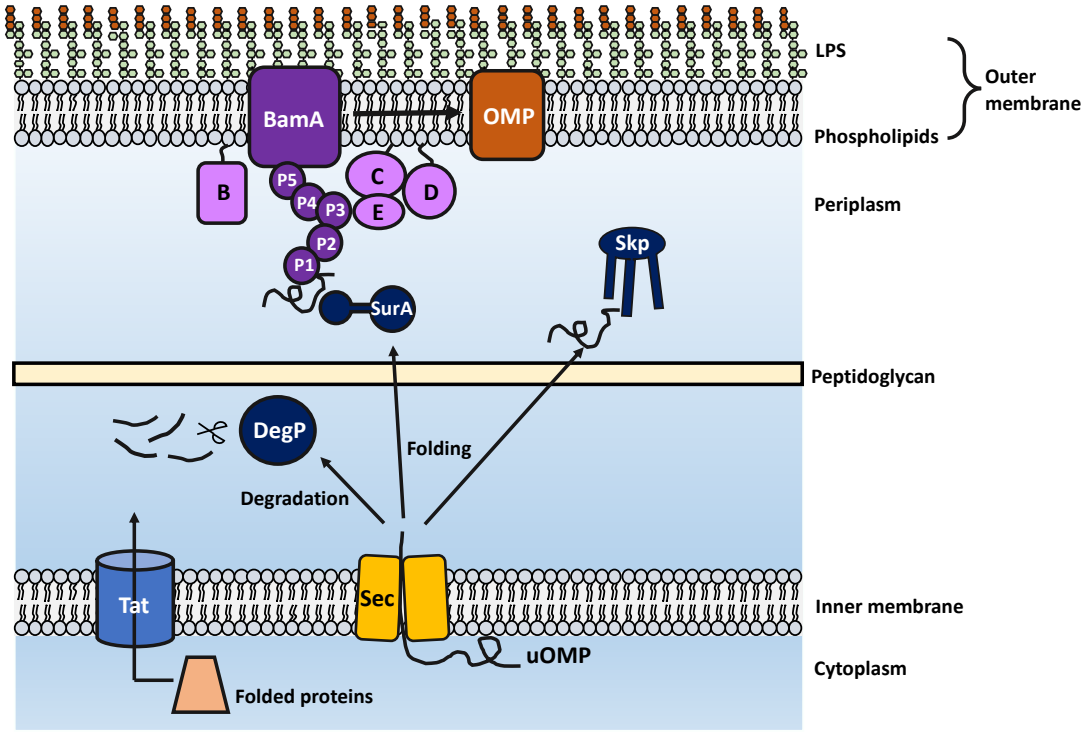


Figure 3. The Gram-negative cell envelope and outer membrane protein biogenesis. The Gram-negative outer membrane is formed of phospholipids on the inner leaflet and LPS on the outer leaflet. Unfolded outer membrane proteins (uOMPs) are transported from the cytoplasm to the periplasm via the Sec machinery located within the inner membrane. uOMPs are transported to the β-barrel assembly machinery (BAM) complex via the periplasmic chaperones Skp and SurA. The BAM complex is formed for five subunits, BamA-E (BamB-E are denoted by single letters in the figure). Misfolded or aggregated uOMPs are degraded by the periplasmic protease, DegP.

1.4.2 Lipopolysaccharide

LPS, located on the outer leaflet of the OM, are crucial to the protective function of the cell envelope and provide a permeability barrier to toxic compounds and resistance to a number of antimicrobials (May and Grabowicz, 2018).

LPS are a key virulence factor of pathogenic bacteria recognised by the host and are therefore classified as PAMPs. To overcome this and evade host immune responses, many pathogenic bacteria have employed mechanisms of host immune evasion via alterations in LPS structure for example, *Yersinia pestis* has been shown to differentially modulate its lipid A acylation, resulting in a less immunogenic LPS, at host body temperatures in order to evade the immune system (Matsuura, 2013). *Salmonella* also possesses the ability to modify its extracellular LPS structures in response to the host environment and the presence of cationic antimicrobial peptides

(AMPs). Studies have shown that increased O-antigen length can increase complement resistance and reduce the rate of *Salmonella* uptake by macrophages (Murray et al., 2003, Murray et al., 2006), whilst specific LPS modifications can prevent interactions with positively charged cationic AMP molecules, thereby increasing resistance to this class of antimicrobials (see 1.5.2.2 for further information).

Due to its central protective role in the bacterial cell envelope, monitoring and maintenance of LPS integrity is extremely important. Damage to LPS and the accumulation of LPS components in the periplasm induces both the σ^E envelope stress response (1.5.1.3) and the Rcs stress responses (Klein and Raina, 2019).

1.4.2.1 LPS structure

LPS are formed of three major structural domains: lipid A, the core oligosaccharide and the O-antigen (Maldonado et al., 2016). Lipid A is the major membrane-anchoring component of LPS and is a highly conserved hydrophobic glucosamine disaccharide structure (Raetz et al., 2009). Attached to lipid A is the core oligosaccharide, protruding from the OM. The core oligosaccharide is comprised of an inner core containing 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo, keto-deoxyoctulosonate) and heptose sugars and an outer core formed of hexose sugars, for example D-glucose and D-galactose (Raetz and Whitfield, 2002). Finally, the O-antigen (sometimes referred to as the O-side chain) is attached to the core oligosaccharide and is formed of repeating oligosaccharide subunits (up to 40) each comprised of between 3 and 5 sugar molecules (Lerouge and Vanderleyden, 2002). While the lipid A-Kdo structure is a very highly conserved LPS component across Gram-negative bacterial species, it can undergo modifications under environmental stresses; for example, *Salmonella* lipid A modifications increase resistance to the cationic AMP polymyxin B and reduce host recognition by TLR 4 (Kawasaki et al., 2005). In contrast to lipid A, the O-antigen is the most diverse LPS component, likely because the O-antigen is most commonly involved in direct interactions with the environmental niche of different pathogens (Whitfield and Trent, 2014). Within the *Salmonella enterica* species there are 46 serogroups, each of which have variability in their O-antigen structures (Wang et al., 2002). The selective pressures of different environmental stresses encountered by bacteria, such as the host immune response, ultimately drive the variability of O-antigen polysaccharides (Whitfield and Trent, 2014). It should be noted that the O-antigen is not conserved across all bacterial species, notable examples lacking this component are the pathogens *Neisseria meningitidis* and *Bordetella pertussis*, in addition to the laboratory *E. coli* K12 strain (Sperandeo et al., 2017a).

LPS molecules are highly negatively charged and repulsions between molecules has a negative impact on their ability to closely pack at the OM and provide a highly tight barrier. However, to enable their tight packing, divalent cations (namely Mg^{2+} and Ca^{2+}) intercalate between LPS molecules and enhance their packing and overall protection of the OM (Clifton et al., 2015).

1.4.2.2 LPS biosynthesis

LPS synthesis is reviewed in detail in Whitfield and Trent (2014) and will be described in brief here for context of this thesis.

Much like the majority of our understanding of the Gram-negative cell envelope, our current knowledge of LPS biosynthesis is primarily derived from studies in *E. coli* (Sperandeo et al., 2017a). Lipid A synthesis is defined as the Raetz pathway (Raetz et al., 2009) and is initiated at the cytoplasmic leaflet of the inner membrane, where the majority of the LPS biosynthetic machinery is bound or associated (Raetz et al., 2009, Klein and Raina, 2019). Biosynthesis of lipid A is combined with the initial process of core oligosaccharide synthesis, which results in the formation of a lipid A complex bound to two Kdo groups (Whitfield and Trent, 2014). The lipidA-Kdo complex is subsequently transported to the periplasm, across the IM, via an ATP-binding cassette (ABC) transporter called MsbA (Zhou et al., 1998). The O-antigen is synthesised separately to the lipidA-core and is ligated to this complex at the outer leaflet of the IM, within the periplasm (Simpson et al., 2015). Polymerisation of the highly variable O-antigen occurs on a carrier molecule, undecaprenyl phosphate (UDP), and the final O-antigen is ligated to the nascent LPS-core molecule by the ligase WaaL (Han et al., 2012). This process occurs within the periplasm and the mature LPS is then transferred to the LPS transport machinery (Lpt) for passage across the periplasm to the OM (Sperandeo et al., 2017a).

1.4.2.3 LPS transport and machinery

LPS transport from the cytoplasm to the OM is somewhat difficult due to the amphipathic properties of LPS molecules (Sperandeo et al., 2017a). Mature LPS, complete with O-antigen, are transported via the Lpt machinery from the site of assembly at the IM to the OM cell surface. Studies in *E. coli* and *Neisseria meningitidis* have led the way in the characterisation of this process which requires the seven member essential protein complex LptA-G (Bos et al., 2004). The complex spans the periplasm from the IM to the OM but can be divided into two subassemblies, the $LptB_2FG$ complex at the IM and the LptACDE complex at the OM (Figure 4). The $LptB_2FG$ assembly is an ABC transporter and is responsible for removing LPS from

the IM, driven by the ATPase activity of the complex (Ruiz et al., 2008, Whitfield and Trent, 2014). The IM-anchored protein LptC forms the first component of the OM subassembly and although lacking in ATPase activity it still contributes to extraction of LPS from the IM (Sperandeo et al., 2017b). Once detached from the IM, LPS molecules are transferred in an ATP-dependent process from $LptB_2FG$/LptC to LptA in the periplasm (Okuda et al., 2012). LptA forms a 'bridge' between the IM- and OM-associated assemblies of the Lpt machinery, interacting with LptC at its N-terminal domain and LptD at its C-terminal domain (Bowyer et al., 2011). *In vitro,* LptA has been observed to form oligomeric structures; however, it remains unclear whether this is the case *in vivo* and moreover, exactly how many LptA molecules form the periplasmic bridge (Sperandeo et al., 2017a).

In the final step of their transport, LPS molecules are accepted from LptA by the LptDE protein complex in the OM, for insertion into the outer leaflet. LptD is a β-barrel protein while LptE is a lipoprotein, which is located within the LptD barrel (Chng et al., 2010, Chimalakonda et al., 2011). This translocon-forming structure is described as a "plug-and-barrel" architecture (Freinkman et al., 2011). During transfer from LptA, the lipid A component of the LPS molecule is transferred to LptD while the saccharide components (core and O-antigen) pass through the hydrophilic barrel of the LptDE translocon. LptE is predicted to interact with the negative charges of LPS molecules and prevent LPS aggregation during their assembly at the OM. The contribution of LptE is deemed less important than LptD in the receipt and subsequent insertion of LPS into the OM, particularly in bacterial species whose LPS lack the negatively charged O-antigen chain (Bos and Tommassen, 2011, Sperandeo et al., 2017a). In this case, such as in *N. meningitidis*, it is understood that the major role of LptE is to function as a chaperone for LptD and survival of Δ*lptE* mutants in these species are not severely affected, unlike deletion mutants lacking any of the other six Lpt components (Bos and Tommassen, 2011).

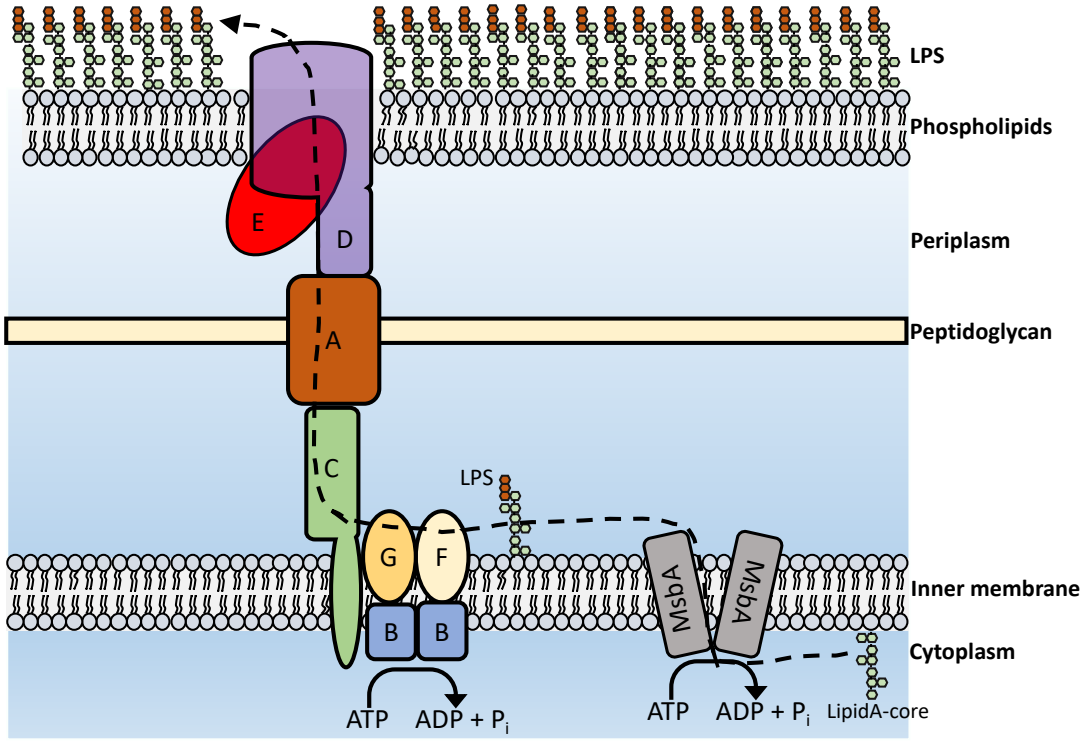


Figure 4. The LPS transport (Lpt) machinery of Gram-negative bacteria. The lipidA-core oligosaccharide complex is synthesised on the inner leaflet of the IM before transport to the outer leaflet via the ABC-transporter MsbA, powered by the hydrolysis of ATP. Addition of the O-antigen occurs in the periplasm by the WaaL machinery (not shown). The periplasm-spanning Lpt machinery transports the mature LPS to the cell surface for insertion on the outer leaflet of the OM. Each Lpt component is denoted by a single letter in the figure. Adapted from Okuda et al. (2016), Bertani and Ruiz (2018).

1.4.3 The Gram-negative envelope as a therapeutic target

Since the Gram-negative envelope is a crucial barrier between the intracellular and extracellular space and maintenance of this barrier is essential for cell viability, the envelope is of great interest for the development of novel antimicrobials.

As indicated above, components of the BAM complex, periplasmic chaperones and LPS have all been linked to pathogenesis in a number of bacteria, including *Salmonella*. BAM complex inhibitors can be considered as highly effective novel therapeutics. Multi-drug resistance (MDR) efflux pumps, located within the OM, are one of the major contributors to bacterial antimicrobial resistance. Therefore, it is suggested that targeting specific processes (such as OMP assembly) at the OM rather than intracellular processes may be a more effective strategy (Hart et al., 2019). Several studies have identified small molecules, peptide fragments of BAM complex components themselves and antibodies that target BAM components

(primarily BamA) that lead to growth defects and increased sensitivity to antimicrobials including vancomycin and rifampicin (Hagan et al., 2015, Storek et al., 2018, Hart et al., 2019). In addition to the BAM complex, targeting the LPS biogenesis and transport machinery has also been explored; Vetterli et al. (2018) reported identification of a peptide with antimicrobial activity towards *E. coli*, *S*. Typhimurium and *Klebsiella pneumoniae* that targets LptA and LptD leading to OM biogenesis defects.

Clearly, the bacterial envelope is an essential component for cell viability and processes within play important roles in pathogenesis. A continued understanding of its biogenesis and maintenance, particularly in the presence of antimicrobials and within a host environment, is essential should novel treatments and vaccines be discovered.

1.5 Bacterial stress responses

The bacterial lifecycle is accompanied by a multitude of stressors including, but not limited to, extreme changes in temperature and pH, reactive oxygen species and nutrient starvation, all of which can lead to lethal damage to cellular proteins and membranes. Bacterial stress responses (both cytoplasmic and envelope) are induced during stress and lead to the induction of a complex network of regulatory pathways, enabling the expression of genes required to respond to disruptions in cellular homeostasis. Stress responses can be grouped into sigma (σ) factors and two component systems (TCS). Much of the work presented here has arisen from studies in the model organism *E. coli* and due to their close similarities, many of the processes are well conserved in *Salmonella*. However, key differences between these bacteria and their stress response regulons do exist, highlighting the need for extending studies beyond *E. coli*.

1.5.1 Sigma factors

1.5.1.1 σ^S

Often referred to as the general stress response, σ^S is highly expressed during stationary phase growth (Battesti et al., 2011). In addition to its growth phase dependent expression, further studies have identified that *rpoS* is induced under nutrient starvation, high/low pH, high/low temperature, high/low osmolarity, DNA damage, oxidative stress, near-UV irradiation and ethanol stress (Battesti et al., 2011). The σ^S regulon is vast and approximately 10% of the *E. coli* genome is understood to be under its direct and indirect control (Weber et al., 2005), although

later studies have stated that at least 20% of the genome may be regulated by σ^S (Wong et al., 2017). Studies in other bacterial species, including *Salmonella*, have identified unique members of the regulon (Ibanez-Ruiz et al., 2000), emphasising the need for study across multiple bacteria.

RpoS is involved in cross-talk with other stress response systems including CpxAR (Kato et al., 2012), Rcs (Peterson et al., 2006) and PhoPQ (Bougdour et al., 2008). With such a large regulon of genes, in addition to the cross-talk with other envelope stress responses (ESRs) each with their own expansive regulons, RpoS is under strict transcriptional, translational and post-translational control.

1.5.1.1.1 σ^S transcriptional control

Expression from the *rpoS* gene is controlled by multiple mechanisms including promoters, small molecules and trans-acting factors (Hengge-Aronis, 2002). With multiple complex levels of control, the exact mechanisms regulating *rpoS* expression are still not entirely understood. In terms of transcriptional regulation via a promoter, the two component system BarA-UvrY positively regulates *rpoS* expression in *E. coli* (Mukhopadhyay et al., 2000, Pernestig et al., 2001) and this is homologous to the GacA-GacS two component system in *Pseudomonas* (Venturi, 2003). Moreover, in *Salmonella* the global regulator Fis prevents *rpoS* transcription via direct interactions with the *rpoS* promoter. Fis is highly expressed during exponential growth, with no expression during stationary phase (Battesti et al., 2011), therefore enabling specific growth phase-dependent control of *rpoS* expression (Hirsch and Elliott, 2005). When activated by phosphorylation, the response regulator ArcA of the ArcBA TCS is also responsible for negative regulation of *rpoS* expression (Mika and Hengge, 2005).

In addition to transcriptional regulators, the small molecule guanosine pentaphosphate (ppGpp), whose cellular levels increase during nutrient and amino acid limitation/starvation, has also been linked to control of *rpoS* at the transcriptional level, although current understanding is that this is via maintenance of mRNA stability, rather than a direct induction of transcription (Lange et al., 1995, Hirsch and Elliott, 2002, Battesti et al., 2011). Additionally, the global transcriptional regulator cAMP-CRP (cyclic adenosine monophosphate-cyclic adenosine monophosphate receptor protein) negatively regulates *rpoS* during exponential phase growth; *E. coli* Δ*cya* and Δ*crp* mutants (unable to produce cAMP or CRP, respectively) show increased σ^S levels in the exponential phase (Lange and Hengge-Aronis, 1994).

1.5.1.1.2 σ^S translational control

Translational control of RpoS production is evidenced by studies identifying that non-stressed cells can produce constant levels of *rpoS* mRNA yet the RpoS protein is undetectable (Lange and Hengge-Aronis, 1994). The *rpoS* mRNA forms a secondary structure within the 5' UTR, the folding of which prevents ribosomal access (Brown and Elliott, 1997) RpoS translation is initiated via interactions of several sRNAs with the mRNA hairpin, leading to destabilisation and translation (Battesti et al., 2011). sRNAs usually bind to specific mRNA targets and can increase/decrease mRNA stability or inhibit mRNA translation, thereby adding a post-transcriptional level of control over gene activation or repression (Wassarman, 2002). The sRNAs DsrA, RprA and ArcZ are positive regulators of *rpoS* translation and their pairing with the mRNA hairpin leads to hairpin opening and exposure of the ribosome binding site (RBS) for translation to occur (Majdalani et al., 1998, Majdalani et al., 2002, Mandin and Gottesman, 2010, Battesti et al., 2011). Each of the sRNAs inducing RpoS translation are dependent on the RNA chaperone Hfq for their function (Battesti et al., 2011). RpoS translation is also negatively controlled by the OxyS sRNA, the expression of which is controlled by the OxyR transcriptional regulator, induced by H_2O_2 oxidative stress (Mika and Hengge, 2014). Whether OxyS directly interacts with the *rpoS* 5' UTR hairpin is unclear. Instead, it is suggested that OxyS binds to Hfq, preventing it from stabilising DsrA, RprA and ArcA (Hussein and Lim, 2011, Updegrove and Wartell, 2011). Henderson et al. (2013) also inferred that OxyS may interact with *rpoS* mRNA in an Hfq-dependent manner and recruit RNase E for mRNA cleavage.

1.5.1.1.3 Proteolytic control of σ^S

With a maximum half-life of only a few minutes under non-stressed exponential growth conditions, there is a continual and rapid turnover of RpoS molecules within the cell (Zhou and Gottesman, 1998). Exposure to sudden stresses lead to the greatest changes in the proteolysis of RpoS, enabling a fast response to the imposing stress (Hengge-Aronis, 2002). In particular, during nutrient (carbon, magnesium, phosphorus) starvation and stationary phase growth, there is little degradation of RpoS (Battesti et al., 2011). Under non-stressed conditions, RpoS is degraded by the ClpXP ATPase, in concert with the adaptor protein RssB (MviA in *S.* Typhimurium) (Schweder et al., 1996, Bearson et al., 1996, Zhou et al., 2001a). Due to its central role in RpoS turnover, the expression of RssB is under strict control. In its role as a negative regulator of σ^S, the ArcBA TCS is predicted to be responsible for phosphorylation and activation of RssB (Mika and Hengge, 2005). Meanwhile, the

inhibitor of RssB activity (Ira) proteins IraP, IraM and IraD are important in preventing RssB activity and therefore delivery of RpoS to the ClpXP pathway (Bougdour et al., 2006, Bougdour et al., 2008).

1.5.1.1.4 σ^S and *Salmonella* pathogenesis

As a general stress response regulator, σ^S induction allows for the protection against multiple stressors, particularly for pathogenic bacteria during host infection. In *S.* Typhimurium, the *Salmonella* virulence plasmid genes (*spv*) are under σ^S control (Fang et al., 1992, Norel et al., 1992). These genes are required for systemic infection with *spv* mutants attenuated for virulence in mice, with an inability to colonise the spleen and Peyer's patches (Nickerson and Curtiss, 1997). The precise role of σ^S in *S.* Typhi virulence is less clear. Despite this, RpoS has been attributed to *S*. Typhi proliferation in macrophages; Alam et al. (2006) and Velásquez et al. (2016) identified that SPI-9 operon genes – which are involved in adhesion to epithelial cells – are positively regulated by σ^S in response to acidic pH and high osmolarity.

1.5.1.2 σ^H

σ^H, encoded by the *rpoH* (formerly *htpR*) gene, was the first alternative sigma factor to be identified in *E. coli* (Straus et al., 1987, Zhao et al., 2005). σ^H plays a major role in the heat shock response, induced by extreme changes in temperature (Straus et al., 1987). However, as the known regulon expands additional inducing cues are being identified, including sublethal ethanol exposure and oxidative stress, and much of the regulon also overlaps with the σ^{70} (σ^D, RpoD) general housekeeping sigma factor (Bang et al., 2005, Nonaka et al., 2006). Genes induced by σ^H generally function to protect cytoplasmic proteins and DNA and include small heat shock proteins (sHsps), chaperones, proteases. Although σ^H is primarily attributed to maintaining cytoplasmic protein homeostasis, more recent work has identified roles in the maintenance of membrane homeostasis, alongside the two component system CpxAR (1.5.2) (Nonaka et al., 2006). This is, in part, due to the fact that 25% of the σ^H regulon encodes for proteins localised in the inner membrane (Nonaka et al., 2006). Further studies are required to precisely elucidate the contribution of σ^H and σ^H-regulated proteins to the maintenance of membrane/envelope homeostasis. In addition to chaperones and proteases, there is a prominence of transcription factors within the σ^H regulon, including RpoD, PhoPQ, ZntR, Mlc and NarP, many of which respond to extracytoplasmic stress conditions (Nonaka et al., 2006).

With such an expansive regulon, the activity of σ^H is under strict control; this is exerted both post-transcriptionally and post-translationally, although the majority of its

regulation is performed by the latter (Straus et al., 1987). In support of this, studies have identified that at *rpoH* induction temperatures, the amount of the RpoH protein substantially increases even though the rate of *rpoH* mRNA synthesis remains constant, thereby indicating a preference for post-translational control (Erickson et al., 1987).

1.5.1.2.1 Post-transcriptional control of σ^H

The *rpoH* mRNA forms a secondary structure within the 5' end commonly known as an RNA thermometer (Nagai et al., 1991, Yuzawa et al., 1993, Morita et al., 1999). RNA thermometers allow for temperature dependent transcription in which the mRNA is folded in a certain way at low temperatures, usually blocking access to the Shine-Dalgarno sequence or the start codon and inhibiting ribosomal access (Kortmann and Narberhaus, 2012). RNA thermometers have been found to control the expression of sHsps in multiple bacterial species (see Chapter 3) and the *rpoH* RNA thermometer was the first identified example of this phenomenon (Roncarati and Scarlato, 2017). Uniquely, RNA thermometers may provide a potential route for novel therapeutics; Barros et al. (2016) described a mechanism of exploiting these structures in which small molecules were developed to stabilise the RNA thermometer, resulting in no translation of heat shock proteins during stress. However, whether this therapeutic method can substantially reduce bacterial transmission via food products and survival within the host environment remains to be determined.

1.5.1.2.2 Post-translational control of σ^H

The σ^H protein is highly unstable, with a half-life of only 1-2 mins (Bittner et al., 2017) and its intracellular concentration is tightly controlled by proteases and cytoplasmic chaperones, both of which are dependent on a specific set of amino acids in RpoH, termed the region of homeostatic control (Yura et al., 2007, Lim et al., 2013). Under non-inducing conditions, the membrane-bound protease FtsH degrades σ^H and maintains low intracellular levels. In *E. coli ftsH* mutants, the level of σ^H and expression of σ^H-regulated genes (i.e. the heat shock response) are both elevated (Tomoyasu et al., 1995). Moreover, the ATP-dependent protease ClpQY is also involved in maintaining σ^H concentration and its overexpression can alleviate the effects of an *ftsH* deletion (Kanemori et al., 1997).

The σ^H-regulated ATP-dependent chaperones DnaK, DnaJ, GrpE and GroEL/S control σ^H stability and activity via a negative feedback loop (Straus et al., 1990, Tomoyasu et al., 1998, Guisbert et al., 2004, Zhao et al., 2005). Under non-inducing conditions, the chaperones bind to σ^H and deliver it to the FtsH protease for

degradation (Figure 5). However, these cytosolic chaperones also play important roles in repairing or delivering misfolded proteins to degradation machinery, particularly when the cell is under stress, thereby preventing σ^H-FtsH interactions. In such conditions, e.g. elevated temperature, the chaperones bind instead to other misfolded/damaged proteins and are titrated away from σ^H (Arsène et al., 2000).

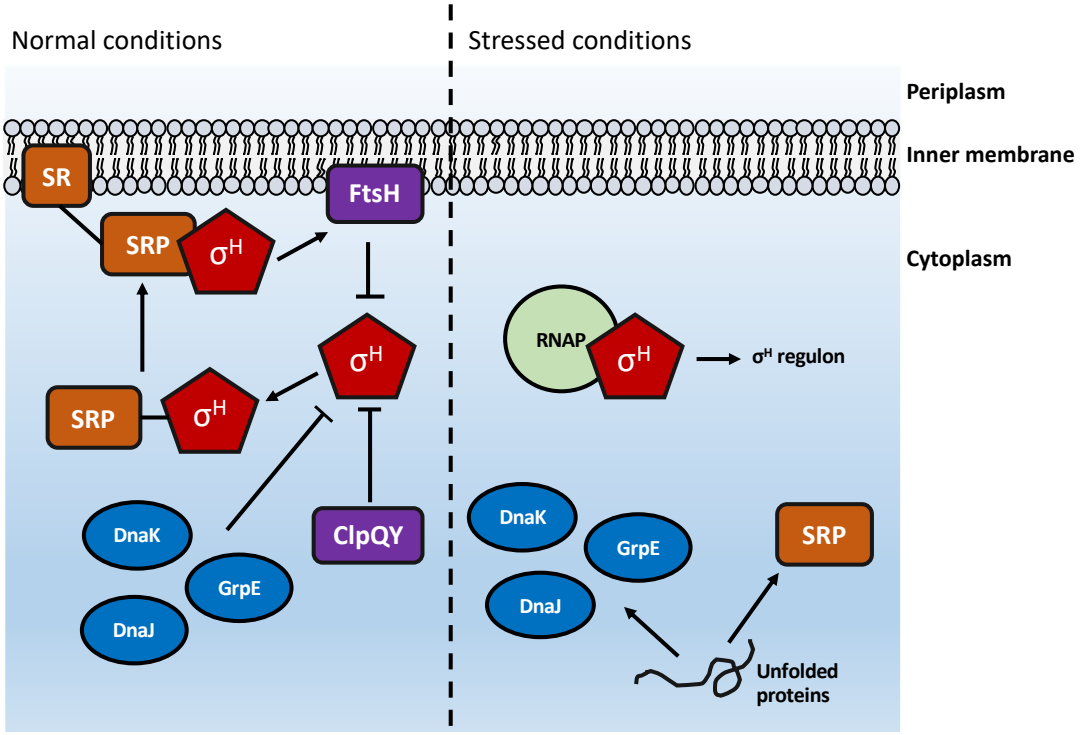


Figure 5. A complex regulatory network controls σ^H activity under non-stressed (normal) conditions. Under non-stressed conditions, the signal recognition particle (SRP) recruits free σ^H in the cytoplasm and transports it to the IM, where σ^H is degraded by FtsH. The ClpQY ATP-dependent protease is also responsible for control of σ^H cytoplasmic levels, degrading the sigma factor in the cytoplasm. In addition to SRP, the cytoplasmic chaperones DnaK, DnaJ and GrpE bind to σ^H and deliver it to the FtsH protease for degradation. Under stressed conditions (e.g. heat shock), the concentration of damaged/unfolded proteins in the cytoplasm increases, titrating the DnaKJ/GrpE chaperone triad and SRP away from σ^H. The free sigma factor can then associate with RNA polymerase (RNAP) and induce the transcription of a vast regulon of heat shock proteins, chaperones, proteases and additional transcriptional regulators.

The negative feedback loop is mediated by the signal recognition particle (SRP) and the SRP receptor (SR), which usually transports proteins to the inner membrane, and it has been shown that SRP interacts with σ^H in the region of homeostatic control (Lim et al., 2013). Lim et al. (2013) identified that σ^H is associated with the inner membrane via SRP and thus is able to manage protein homeostasis both within the cytoplasm

and the inner membrane. In the same study, FtsH degradation was shown only to occur when σ^H was located at the IM. Artificial tethering of σ^H to the inner membrane has been shown to restore a defective σ^H response in σ^H point mutant strains or ΔSRP strains. This is an interesting first identification of IM localisation being required for sigma factor activity, as opposed to inactivity as is the case for the extracytoplasmic sigma factor, σ^E (1.5.1.3).

1.5.1.2.3 σ^H and *Salmonella* pathogenesis

The primary role of σ^H is in the induction of the bacterial heat shock response. The ability of *Salmonella* to survive against changing temperatures is essential for survival both within the food chain and within different hosts with varying body temperatures. As a zoonotic pathogen, *Salmonella* serovars can readily pass between avian and human hosts, with body temperatures of 42 °C and 37 °C respectively. Additionally, temperature sensing is an important mechanism utilised by *Salmonella* for the environmental control of the expression of virulence genes; for example, exposure of *Salmonella* to 42 °C induces expression of SPI-2 and SPI-5 but represses SPI-1 (Sirsat et al., 2011). The heat shock response and small heat shock proteins (sHsps) are introduced in greater detail in Chapter 3. However, it is important to note here that despite their description as sHsps, an emerging idea is that these proteins and the σ^H stress response are not only involved in survival against heat stress. For example, in *E. coli* σ^H has been shown to induce the expression of the *phoP* and *phoQ* genes, which encode the PhoPQ two-component system and contribute to bacterial resistance to antimicrobial peptides and in *Salmonella,* the ability to survive in the SCV (Nonaka et al., 2006). Further to this, sHsps have also been directly linked to general protein folding and they are predicted to contribute to survival in a wider variety of conditions and also during pathogenesis (Guisbert et al., 2004). Moreover, as is a common theme for much of our knowledge about stress responses, our core understanding of the σ^H response and sHsps has arisen following characterisation in *E. coli*. In comparison to *E. coli*, there is certainly a distinct lack of studies that focus on the specific contributions of σ^H-regulated proteins to *Salmonella* survival and pathogenesis.

1.5.1.3 σ^E

The extracytoplasmic sigma factor, σ^E (*rpoE*) is responsible for much of the maintenance of cell envelope homeostasis. This ESR primarily senses misfolded OMPs in the OM or periplasm, in addition to mislocalized LPS and their constituents. The σ^E pathway is induced by oxidative stress, heat shock, carbon starvation, biofilm

formation, acid stress, ultraviolet A radiation, P22 phage and hypo-osmotic shock (Testerman et al., 2002, Bang et al., 2005, Rowley et al., 2006, Muller et al., 2009, Amar et al., 2018, Hews et al., 2019a). Induction of the σ^E ESR occurs via a series of proteolytic cleavage events (regulated intramembrane proteolysis, RIP), culminating in the release of σ^E from its covalently bound inhibitor, enabling the sigma factor to interact with RNA polymerase and control the expression of a vast regulon of genes.

σ^E-regulated genes are involved in OM biogenesis and maintenance, in addition to virulence in a range of bacterial pathogens including *Salmonella*. The *Salmonella* σ^E regulon was previously identified by Skovierova et al. (2006) and within this regulon are a number of genes of unknown function and genes specific to *Salmonella* sp. Since a large majority of current knowledge surrounding the σ^E ESR and its regulon is derived from studies in *E. coli*, an expanded knowledge of σ^E-regulated genes in *Salmonella*, particularly those which are specific to this species, will certainly enhance our understanding of *Salmonella* survival during stress. Of interest to this thesis are four σ^E-regulated genes, whose regulation is shared with σ^H and of two of which are unique to *Salmonella*. While these genes will be introduced in detail in Chapter 3, in this section the mechanisms of σ^E activation, regulation and overall known contributions to *Salmonella* pathogenesis will be described.

1.5.1.3.1 Activation of the σ^E response

σ^E is encoded by *rpoE*, an essential gene in *E. coli* and *Yersinia sp* (De Las Peñas et al., 1997a, Heusipp et al., 2003). Studies have identified a small number of suppressor mutants in *E. coli* that have enabled the generation of *rpoE* mutants; insertion mutations in *ydcQ*, a putative DNA binding protein (Button et al., 2007), *yhbW* and *ptsN*, genes of unknown function (Hayden and Ades, 2008) allow for an *rpoE* deletion to be tolerated. Interestingly, despite close similarities to *E. coli*, *rpoE* is not an essential gene in *Salmonella* (Humphreys et al., 1999, Skovierova et al., 2006). Recent studies have identified that *rpoE* deletions in *Salmonella* mutants that have also the LPS O-antigen are lethal, suggesting that usually when present, the O-antigen provides a level of protection to the *Salmonella* OM and envelope, thus reducing the detrimental effect of an *rpoE* deletion and allowing it to be tolerated (Amar et al., 2018).

The RIP cascade of σ^E activation is presented in Figure 6. Under non-inducing conditions, σ^E-RNAP interactions are sequestered two inhibitors, RseA and RseB, both of which are encoded within the σ^E operon (Dartigalongue et al., 2001). Whilst the transmembrane protein RseA is the primary inhibitor, RseB binds to RseA and

increases affinity of the complete inhibitor complex to σ^E (De Las Peñas et al., 1997b, Collinet et al., 2000) (Figure 6). During stress or unfavourable conditions, the C-terminal region of misfolded proteins in the periplasm interact with the PDZ domain of DegS. DegS is a site-1 protease (S1P) located within the inner membrane and interactions with uOMPs induce a conformational change that leads to the exposure of the protease domain for cleavage of RseA, releasing the RseB-bound component of RseA into the periplasm (Ades et al., 1999, Alba et al., 2002, Kanehara et al., 2002, Li et al., 2009, Chaba et al., 2011, Kim, 2015). Subsequently, the site-2 protease (S2P) RseP (YaeL) is recruited which cleaves the cytoplasmic domain of RseA, resulting in the release of the RseA-σ^E complex into the cytoplasm. Despite this mechanism of RseA cleavage currently viewed as the canonical pathway, additional studies have identified a non-canonical mechanism of σ^E pathway activation which occurs independently of DegS (Rowley et al., 2005, Muller et al., 2009). Specifically, this non-canonical pathway is uniquely activated during acid stress (Chapter 4).

Following release of RseA-σ^E from the IM and into the cytoplasm, the SspB adaptor binds to the complex and enables recruitment of the ClpXP protease (Flynn et al., 2004, Baker and Sauer, 2012). Finally, ClpXP cleaves the remaining fragment of RseA bound to σ^E and results in release of the free sigma factor for binding to RNAP and induction of the σ^E regulon (Chaba et al., 2007).

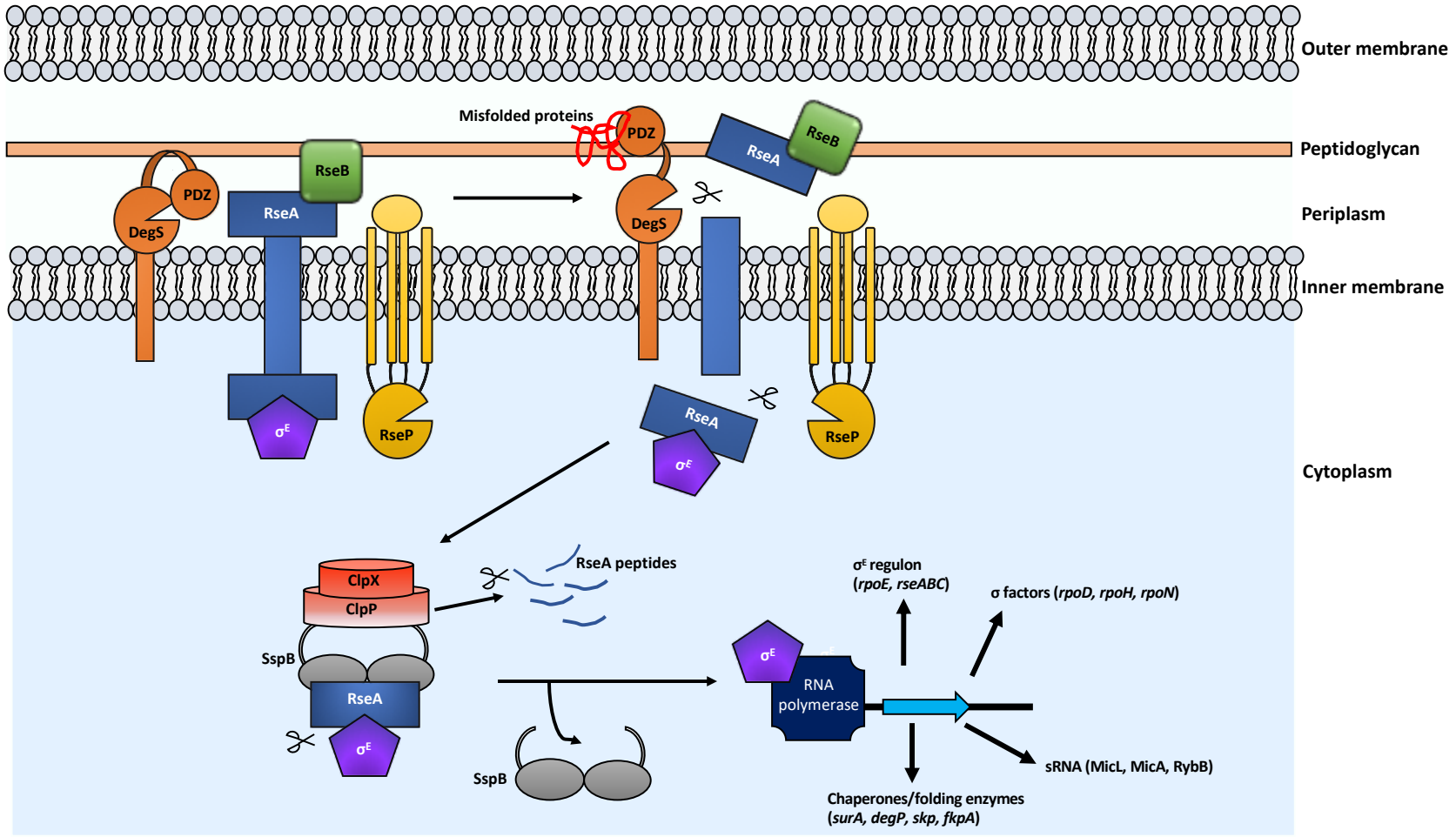


Figure 6. The extracytoplasmic envelope stress response σ^E pathway is activated by a series of proteolytic cleavage events. The primary activator of the σ^E cascade is the presence of misfolded proteins accumulating in the periplasm. Downstream proteolysis results in the release of free σ^E which associates with RNA polymerase and induces transcription of a vast regulon of genes. Taken from (Hews et al., 2019).

1.5.1.3.2 σ^E and sRNAs

The σ^E regulon encodes three regulatory sRNAs (MicA, RybB and MicL) involved in the regulation of the σ^E response (Vogel and Papenfort, 2006, Thompson et al., 2007, Udekwu and Wagner, 2007, Guo et al., 2014). The promoters upstream of *micA* and *rybB* are two of the strongest σ^E regulon promoters during exponential phase growth (behind only the *rpoE* promoter itself), while at stationary phase *micA* is the strongest promoter (Mutalik et al., 2009). In the same manner as the σ^S sRNAs described above, MicA, RybB and MicL function in an Hfq-dependent manner. In fact, deletion of *hfq* activates the σ^E ESR through increased RseA cleavage, in a DegS-dependent manner (Figueroa-Bossi et al., 2006).

The overall functions of the σ^E-encoded regulatory sRNAs are to downregulate cellular processes, particularly during stressful/unfavourable growth conditions, including uOMP and lipoprotein production, which if not controlled may lead to continued activation of the σ^E response (Guo et al., 2014) (Figure 7). RybB is an 81 nt transcript, first identified in *E. coli* as highly expressed during stationary phase (Thompson et al., 2007). Similarly, MicA (SraD) was also first identified in *E. coli* as a 70 nt transcript, regulated by σ^E only (Udekwu and Wagner, 2007), which accumulates during stationary phase (Vogel and Papenfort, 2006). Both RybB and MicA are conserved in *Salmonella* (Papenfort et al., 2006) and function to decrease expression of outer membrane porins. Specifically, RybB targets *ompC* and *ompW* mRNA while MicA targets *ompA* mRNA (Udekwu et al., 2005, Johansen et al., 2006, Vogel and Papenfort, 2006). Ultimately, downregulation of major OMP production reduces the demand on the BAM complex during periods of stress, when periplasmic uOMP is likely to be increased in comparison to normal (favourable) physiological conditions. MicA also downregulates the PhoPQ two component system by targeting the *phoP* gene (Coornaert et al., 2010), highlighting the importance of cross-talk between stress response systems.

MicL (SlrA, suppressing lap RNA) is the most recently identified σ^E-regulated sRNA. MicL is an 80 nt sRNA processed from a primary transcript of 308nt and located within the *cutC* gene (Guo et al., 2014). Previously identified *E. coli cutC* mutant phenotypes, e.g. sensitivity to copper, are attributable to the loss of the MicL sRNA (Chaba et al., 2011). To date, the only known target of MicL is the *lpp* gene, encoding Braun's lipoprotein (Lpp) (Braun, 1975). Lpp is an OM lipoprotein and is the most abundant protein in *E. coli.* It plays a major role in stabilisation of the cell envelope via its OM-peptidoglycan interactions. During stress, MicL targeting of *lpp* reduces demand on the Lol machinery, enabling increased production of other lipoproteins including

BamD and LptE. Arguably, these lipoproteins are in increased demand by the cell during stress due to the increase in uOMPs and LPS components in the periplasm.

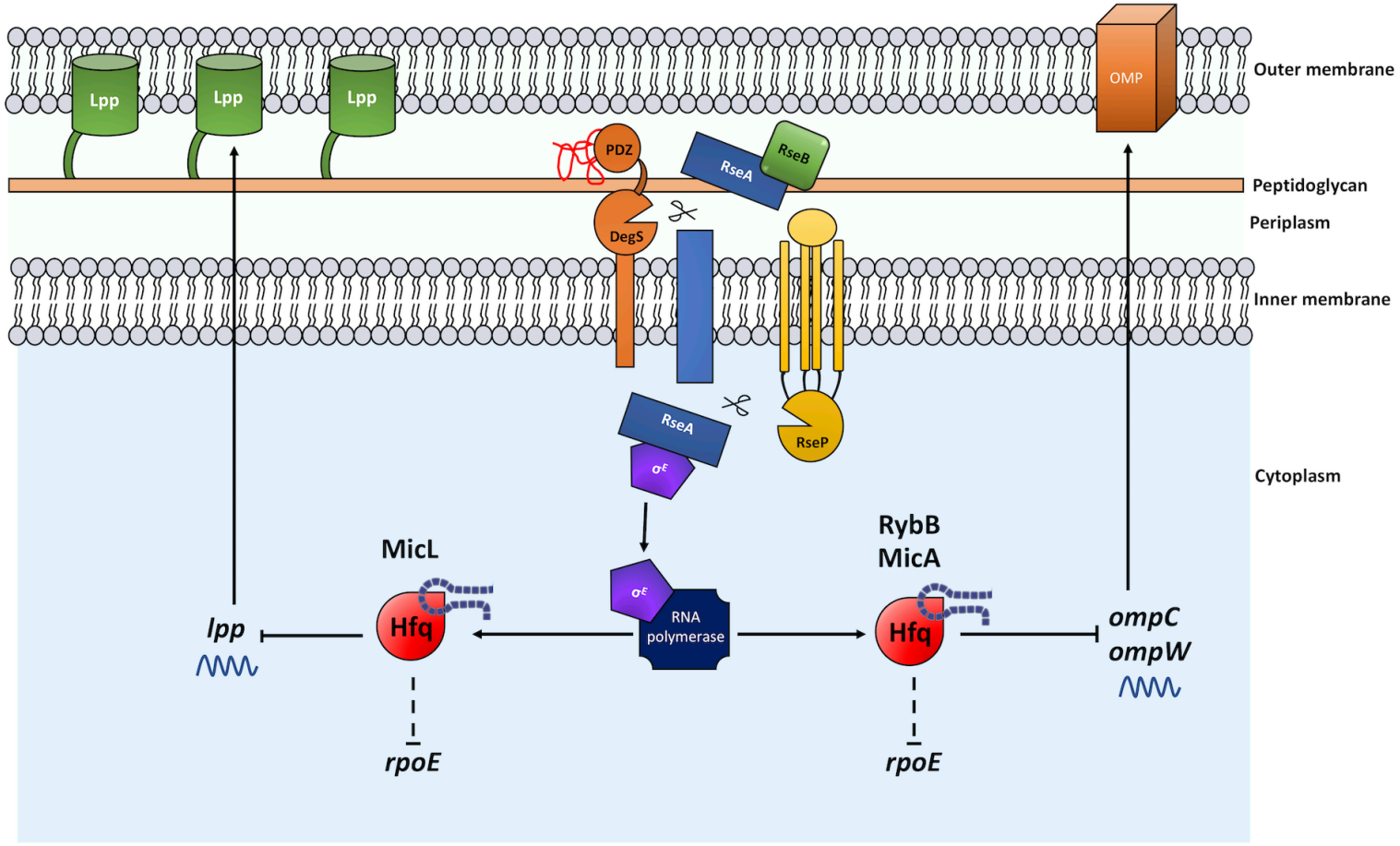


Figure 7. The σ^E-regulated sRNA RybB, MicA and MicL control σ^E activity in an Hfq-dependent manner via the downregulation of the OmpC and OmpW outer membrane proteins (OMPs) and the Lpp lipoprotein. The sRNAs inhibit translation of key OMPs, reducing the overall transport and presence of uOMPs in the periplasm during exposure to stress-inducing conditions. In a negative feedback loop, sRNA also prevent the synthesis of σ^E. Figure taken from Hews et al. (2019a).

1.5.1.3.3 σ^E and *Salmonella* pathogenesis

Members of the σ^E regulon have been implicated at various stages of a *Salmonella* infection (summarised in Figure 8) and deletion of *rpoE* has been shown to attenuate *S.* Typhimurium in the murine infection model (Humphreys et al., 1999). Furthermore, σ^E is essential *in vitro* for the ability of *Salmonella* to survive in the intramacrophage environment (Humphreys et al., 1999) and *rpoE* is upregulated during intracellular survival (Eriksson et al., 2003). Within the macrophage *Salmonella* resides in the SCV in the presence of acidic pH, reactive oxygen species and reactive nitrogen species. σ^E is required for resistance to oxidative stress (Humphreys et al., 1999, Testerman et al., 2002) and has also been shown to be important for acid resistance (Muller et al., 2009) (Chapter 4). Studies focussing on the role of *rpoE* in *S.* Typhi pathogenesis are limited in comparison to those in NTS serovars, likely due to the lack of a specific animal model for the *S.* Typhi serovar. However, expression of both SPI-1 and SPI-2 are reduced in *S.* Typhi *rpoE* mutants (Xie et al., 2016, Zhang et al., 2016), potentially implicating *rpoE* in the environmental control of virulence gene expression.

In addition to RpoE itself, σ^E-regulated chaperones and sRNAs are also implicated in *Salmonella* virulence. Taking this into account, Sydenham et al. (2000) demonstrated that *Salmonella surA* mutants are unable to successfully adhere to and invade epithelial cells and can function as an attenuated live vaccine, protecting against subsequent WT challenge in mice. Whether deletion mutants lacking other periplasmic chaperones pose as potential vaccine strains is unclear, but it has been shown that Skp is required for *Salmonella* murine infection (Rowley et al., 2011). Additionally, *S.* Copenhagen *fkpA* mutants are attenuated for intracellular survival (Horne et al., 1997). Interestingly this phenotype is not conserved in *S.* Typhimurium, unless *fkpA* deletions are combined with *surA* or *degP* deletions (Humphreys et al., 2003).

Finally, in terms of the σ^E-regulated sRNAs, an RNA-sequencing study identified that both MicA and RybB are upregulated inside macrophages, indicating a potential role for virulence (Srikumar et al., 2015). However, their exact contributions have not been confirmed and their upregulation may simply be a downstream effect of increased σ^E expression in this environment.

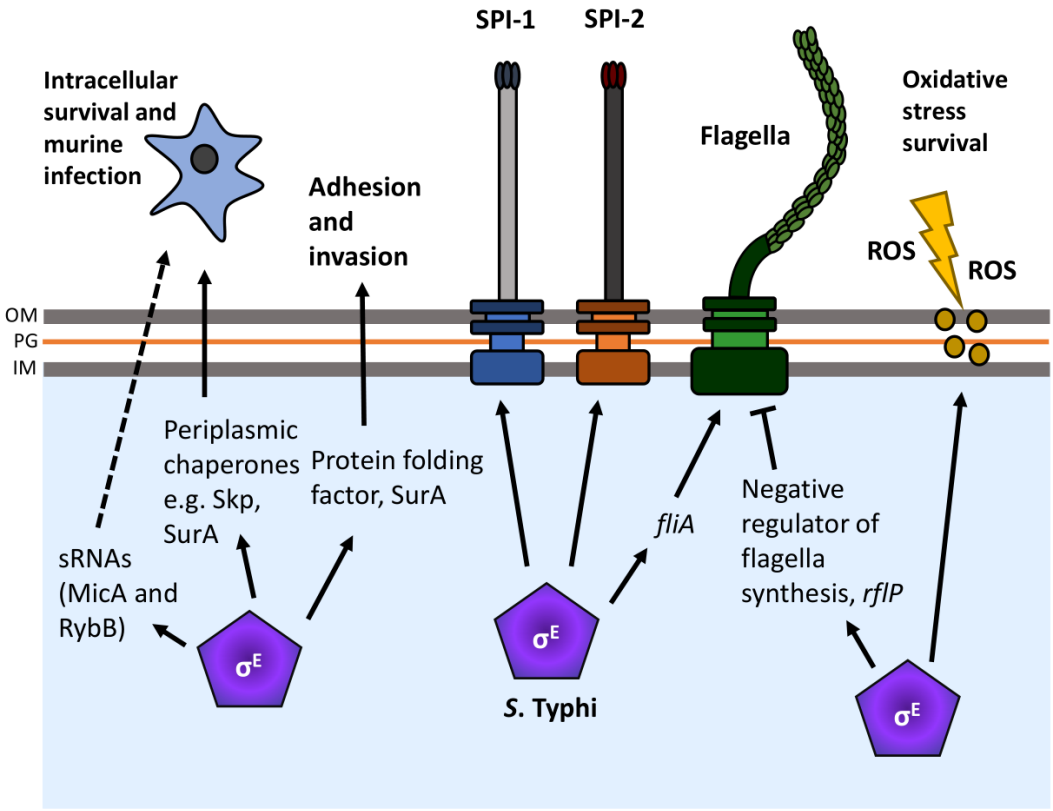


Figure 8. The σ^E response is involved in the regulation of pathogenesis in enteric and systemic *Salmonella* serovars. σ^E and its regulon have been associated with intramacrophage survival and murine infection and are implicated in resistance to oxidative stress. Protein folding factors, for example SurA, controlled by σ^E also contribute to adhesion and invasion capabilities of *Salmonella*. In *S.* Typhi, σ^E has been shown to induce the expression of SPI-1, SPI-2 and the *fliA* gene of the flagella. Figure taken from Hews et al. (2019a).

1.5.2 Two component systems

In addition to sigma factors, two-component systems are commonly used by bacteria to sense and respond to changing and stress-inducing environmental conditions. A canonical two component system consists of a transmembrane histidine kinase and a cytoplasmic response regulator. Typically, additional proteins, often periplasmic, are involved in the regulation and transmission of the cellular response in a TCS.

1.5.2.1 The CpxAR response

The Cpx (conjugative pilus expression) two component system is well conserved across Gram-negative bacteria. In this system, presented in Figure 9, CpxA functions as the histidine kinase, a transmembrane protein located within the inner membrane,

while CpxR is the cytoplasmic response regulator (Raivio and Silhavy, 1997). A number of inducing conditions have been described to date including alkaline pH (Danese and Silhavy, 1998), adhesion to hydrophobic and host surfaces (Otto and Silhavy, 2002), antimicrobial peptides (Audrain et al., 2013) and copper (Yamamoto and Ishihama, 2006, López et al., 2018). Generally, the Cpx response is involved in monitoring and responding to disruptions in IM homeostasis and the detection of OM and periplasmic proteins (Vogt and Raivio, 2012).

Under non-inducing conditions, the system is repressed through the interaction of CpxP, a periplasmic protein, and the periplasmic domain of CpxA (Raivio and Silhavy, 1997, Raivio et al., 1999). However, under inducing conditions such as those outlined above, CpxP is titrated away from CpxA. The precise mechanism of CpxP removal from CpxA is unclear but understanding thus far indicates that misfolded proteins bind to CpxP, resulting in a conformational change and disrupting the interaction with CpxA (Raivio, 2014). CpxP bound to misfolded proteins then delivers these to periplasmic chaperones and proteases, such as DegP, for proteolysis (Buelow and Raivio, 2005, Isaac et al., 2005). Following release of CpxP, CpxA autophosphorylates via its histidine kinase domain, and transfers a phosphate group to CpxR (Raivio and Silhavy, 1997). Then, as an active response regulator, CpxR induces/represses expression of the CpxAR regulon of genes.

In addition to the Cpx components of the TCS, a further OM-associated protein, NlpE, is also involved in induction of the Cpx ESR. NlpE is understood to function as a surface-sensing protein, particularly abiotic surfaces and its overexpression activates the Cpx response (Otto and Silhavy, 2002).

The Cpx regulon of *E. coli* has been well studied and includes genes that influence membrane integrity and envelope protein folding, antimicrobial resistance, metal resistance (in particular copper) and pathogenesis (Raivio et al., 2013). Moreover, the CpxAR response has been directly linked to *Salmonella* pathogenesis with *cpxA* deletion and *cpxA** (constitutively active histidine kinase) mutants significantly attenuated in mice (Humphreys et al., 2004).

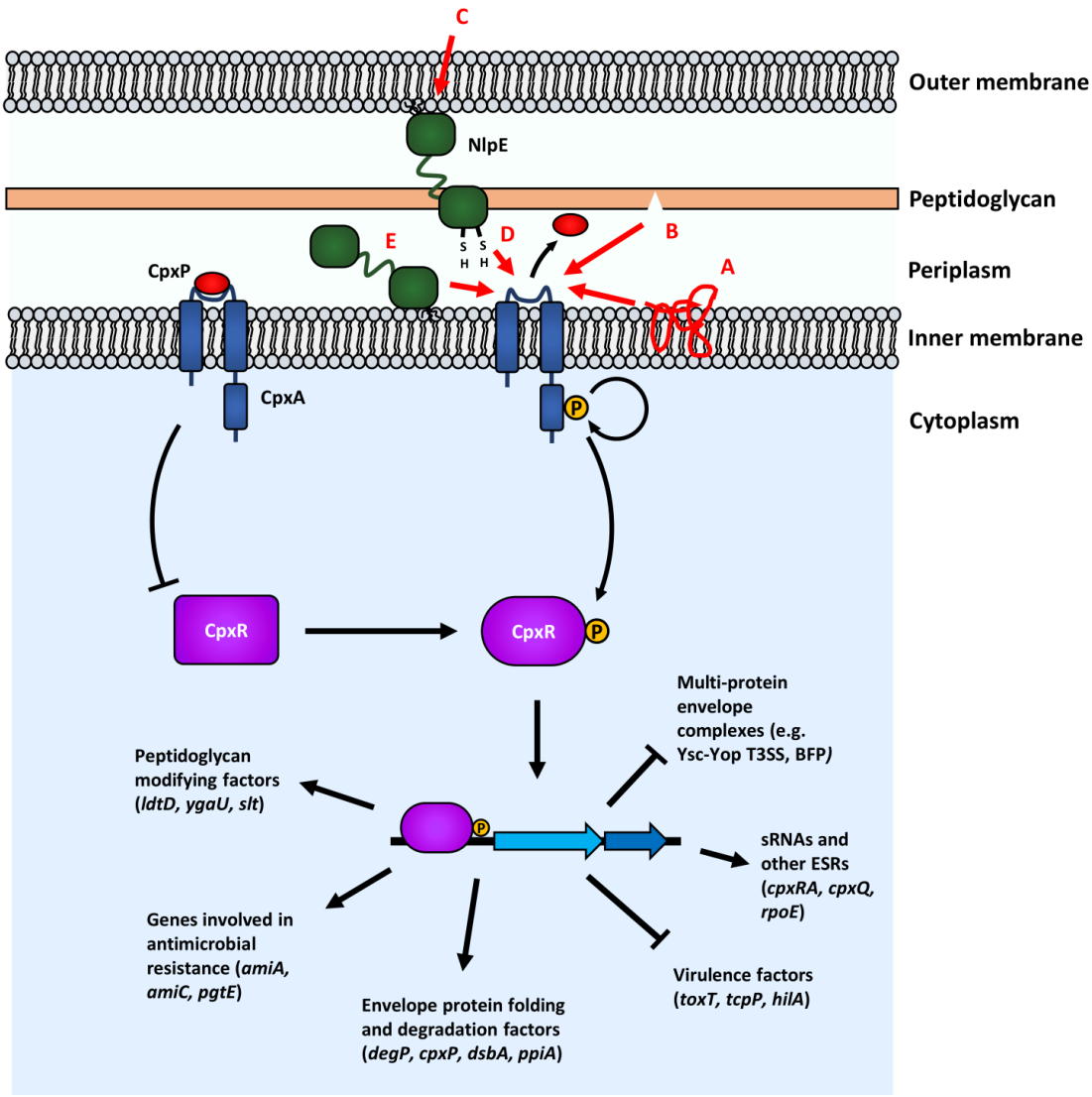


Figure 9. The CpxAR two-component system (TCS) controls genes required for a variety of cellular process including envelope protein folding and antimicrobial resistance. Activation of the Cpx two component system occurs via the outer membrane anchored lipoprotein NlpE, activation of the histidine kinase CpxA and subsequently phosphorylation of the response regulator CpxR. Figure taken from Hews et al. (2019a).

1.5.2.2 The PhoPQ two component system

The PhoPQ TCS was originally named and identified for its regulation of *phoN*, a non-specific acid phosphatase (Kasahara et al., 1992). However, since its initial identification, PhoPQ has been found to respond to low concentrations of divalent magnesium (Mg^{2+}) and acidic pH, both of which are conditions associated with the SCV (Foster and Hall, 1990, Garcia Vescovi et al., 1996, Bearson et al., 1998, Prost et al., 2007).

The PhoPQ TCS features the transmembrane histidine kinase, PhoQ and the cytoplasmic response regulator, PhoP (Prost and Miller, 2008). PhoQ contains a C-terminal histidine kinase and a periplasmic sensing domain capable of detecting the presence of Mg^{2+} (Prost and Miller, 2008). Under low (micromolar) concentrations of Mg^{2+} ions, the PhoP response regulator is phosphorylated and the expression of the PhoPQ regulon is induced. However, when Mg^{2+} is present in millimolar concentrations, the dephosphorylation of PhoP is promoted and PhoP-dependent transcription does not occur (Groisman, 2001). As indicated above, the PhoPQ system is also induced by acidic pH and this will be described in further detail in Chapter 4.

In *Salmonella* the PhoPQ TCS controls a range of genes required for intracellular survival and resistance to cationic antimicrobial peptides (AMPs), including the magnesium transporter genes *mgtABC*, LPS modification machineries (*pagP*, *pbgPE*) and the serum resistance gene *pagC* (Groisman, 2001, Nishio et al., 2005). Moreover, the *prgHIJK* genes required for the formation of the T3SS-1 are repressed by PhoPQ, implicating the TCS in the environmental control of virulence genes (Groisman, 2001). Recent studies have identified that a PhoP activated sRNA, PinT, represses the *hilA* and *rtsA* regulators of the SPI-1 T3SS (Palmer et al., 2019, Kim et al., 2019).

In terms of novel therapeutics, the PhoPQ TCS and two of its regulon members, PagC and PagD, are suggested to be potential vaccine targets for prevention of typhoid fever (Miller et al., 1993).

1.5.2.3 PmrAB

The PhoPQ TCS is closely integrated with a further TCS called PmrAB (Figure 10). The PmrAB TCS was first identified in *Salmonella* in a mutant resistant to polymyxin B, in which a mutation in the PmrA response regulator led to constitutive activation of the system. PhoPQ regulates PmrAB activity (Gunn and Miller, 1996) and this was later shown to be via an indirect mechanism through the positive regulation of *pmrD* (Kox et al., 2000). PmrD is a post-transcriptional regulator of PmrA and increases the stability of phosphorylated PmrA via protein-protein interactions (Kato and Groisman, 2004). Direct activation of PmrAB occurs by acidic pH, low ferric iron and aluminium in *Salmonella* and additionally zinc in *E. coli*. The reasons for the metal induction of PmrAB are unclear and the concentrations required to activate the system exceed any concentration encountered by the bacteria *in vivo.* However, studies suggest that metal sensing may be of importance for bacterial survival in the environment and the

soil, and in the case of *Salmonella*, may prime the bacterium for host infection (Gunn, 2008).

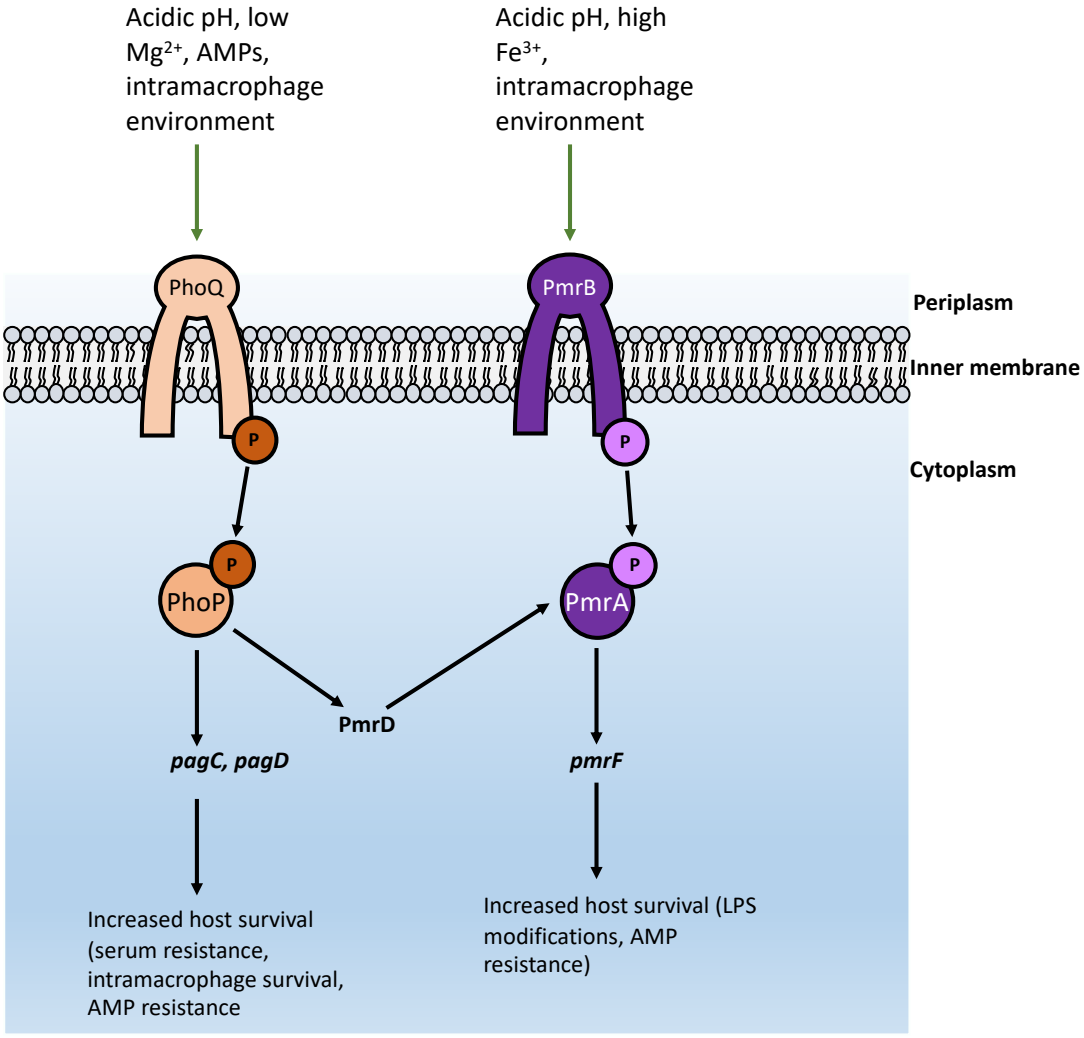


Figure 10. The PhoPQ and PmrAB are interconnected and are involved in *Salmonella* survival in the host. Both systems are induced by the macrophage phagosome and low pH and lead to the modifications of LPS. (Gunn, 2008).

The PmrAB regulon encodes genes primarily involved in the modification of the cell surface LPS. The modifications are dependent on the *pmrA* gene and primarily take the form of the addition of one to two 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (pEtN) moieties (Zhou et al., 2001b). Such modifications result in a change to the overall charge of the cell surface and a reduction in the positive charge of the LPS, thereby reducing the affinity between the bacterium and cationic antimicrobial peptides, driving bacterial antimicrobial resistance. *Salmonella* Δ*pmrF* mutants are unable to produce L-Ara4N and are highly sensitive to cationic AMPs and are attenuated in mice, indicating the vital role of this system *in vivo*. Interestingly,

murine attenuation of Δ*pmrF* was not related to increased killing by cationic AMPs and it is suggested that improper LPS formation results in an altered immune response which in this case, may have resulted in increased killing and clearance of the Δ*pmrF* strain (Strandberg et al., 2012). PmrAB-mediated LPS modifications are not limited to *Salmonella* sp. The system has been attributed to polymyxin resistance and maintenance of OM integrity in a multitude of Gram-negative pathogens including *Klebsiella pneumoniae* (Cheng et al., 2010), *P. aeruginosa* (Moskowitz et al., 2004) and *Citrobacter rodentium* (Viau et al., 2011), commonly used as an *in vivo* model for enterohaemorrhagic *E. coli* (EHEC).

1.6 Thesis Overview

Salmonellae are a major cause of worldwide disease and with antimicrobial resistance continually increasing, there is a pressing need for the identification of novel therapeutic agents and targets. Bacterial stress responses form an essential part of a bacterium's ability to survive within a host and to withstand antimicrobial killing. Therefore, these processes hold great potential in terms of the identification of novel therapeutics. Although the mechanisms and regulons of these responses are well studied, there remains a large majority of regulon members with a poorly described role, particularly in bacterial species other than *E. coli*.

Of primary interest to this thesis is the σ^E response, due to its central role in OM biogenesis and maintenance. Identification of the σ^E regulon revealed a number of proteins of unknown function, many of which are specific to *Salmonella*. This thesis aims to investigate the contribution of four σ^E-regulated proteins (IbpA, IbpB, STM1250 and AgsA, described in detail in Chapter 3) whose contribution to OM homeostasis, *Salmonella* pathogenesis and survival under stressed conditions has not been investigated.

1.6.1 Aims

This thesis aims to advance on current knowledge of poorly characterised σ^E-regulated proteins and understand their contributions to *Salmonella* stress responses and survival. Particular focus was placed on proteins of poorly understood function whose cellular roles (due to shared regulation, the formation of operons and sequence similarities) have been predicted to overlap. These aims were addressed in this thesis through the following points:

- An investigation into the contribution of the poorly characterised σ^E-regulated proteins, IbpA, IbpB, STM1250 and AgsA to *Salmonella* survival against environmental stress (Chapter 3) and more specifically, host-associated stresses including acidic pH and the intramacrophage environment (Chapter 4).
- Identification of protein-protein interactions between IbpA, IbpB, AgsA and STM1250 (Chapter 5).
- Purification of the putative cytoplasmic σ^E-regulated protein STM1250 (Chapter 5).

Chapter 2 Materials and Methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are detailed in Table 1 and Table 2, respectively. In this thesis, the bacterial strains used were derivatives of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) or Escherichia coli (*E. coli*) K12. Mutants were constructed in either the *S*. Typhimurium SL1344 background strain or the *E. coli* K12 MG1655 parent strain. Throughout this work, the isogenic parent stains of each species will be referred to as 'WT'.

2.2 Bacterial culture conditions

2.1.1 Media

Media compositions are detailed in Appendix A. Where required, media were supplemented with antibiotic at the following final concentrations: kanamycin (50 µg/mL), chloramphenicol (30 µg/mL) or ampicillin (100 µg/mL).

2.1.2 Short and long term bacterial storage

For short term storage, bacteria were maintained on LB agar at 4 °C for up to two weeks. For long term storage, bacteria were kept at -80 °C in 25% glycerol or Microbank™ tubes.

2.1.3 Overnight cultures

Overnight (O/N), stationary phase cultures were prepared from short term storage plates (2.1.2). A single colony was used to inoculate 10 mL LB broth (unless otherwise stated) supplemented with antibiotic where required (2.1.1) and cultures were grown at 37 °C with aeration at 200-250 rpm.

2.1.4 Aerobic batch culture

For aerobic growth, 250 mL conical flasks containing 50 mL media were inoculated with O/N culture and grown at 37 °C 220 rpm, unless otherwise stated. Strains harbouring the temperature sensitive plasmid pKD46 were grown at 30 °C.

2.1.5 Anaerobic batch culture

Bacteria were grown anaerobically in minimal M9 media (with supplements detailed in Appendix A) in 200 mL Duran bottles with a silicone lid. Media were sparged with di-nitrogen gas (N_2) for 10 minutes before inoculation with 1 mL O/N culture. Bacteria were grown at 37 °C statically for up to 48 hours.

Table 1. Bacterial strains used in this study.

Strain	Description	Source
***Escherichia coli* strains**		
MG1655	Prototroph; K-12 derivative; *F-*, *lambda*	(Guyer et al., 1981)
TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen™
BL21 DE3	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	NEB™
BTH101	*F- , cya-99, araD139, galE15, galK16, rpsL1 (Str r), hsdR2, mcrA1, mcrB1*	(Karimova et al., 1998, Karimova et al., 2000)
***Escherichia coli* mutant strains**		
Δ*ibpAB*	MG1655 Δ*ibpA*Δ*ibpB* ::Kan	This study
***Salmonella* strains**		
SL1344	*Salmonella enterica enterica* serovar Typhimurium 4/74, *hisG*, *rpsL*	(Hoiseth and Stocker, 1981)
***Salmonella* mutant strains**		
Δ*ibpA*	SL1344 Δ*ibpA* ::Cm	This study
Δ*ibpB*	SL1344 Δ*ibpB* ::Cm	This study
Δ*ibpAB*	SL1344 Δ*ibpA*Δ*ibpB* ::Kan	This study
Δ*STM1250*	SL1344 Δ*STM1250* :Kan	This study
Δ*agsA*	SL1344 Δ*agsA* ::Cm	This study
Δ*ibpAB*Δ*agsA*	SL1344 Δ*ibpA*Δ*ibpB* ::Kan Δ*agaA* ::Cm	This study
Δ*ibpAB* Δ*STM1250*Δ*agsA*	SL1344 Δ*ibpA*Δ*ibpB* ::Kan Δ*STM1250*Δ*agsA* ::Cm	This study
Δ*phoP*	SL1344 Δ*phoP* ::Kan	This study
GVB2551	SL1344 Δ*fkpA*Δ*surA*Δ*ppiAD*	Gift from Professor Mark Roberts, University of Glasgow

Table 2. Plasmids used in this study.

Plasmid	Description	Source
pKD3	pANT-Sγ derivative, FRT-flanked CmR	(Datsenko and Wanner, 2000)
pKD4	pANT-Sγ derivative, FRT-flanked KanR	(Datsenko and Wanner, 2000)
pKD46	Lambda-Red helper plasmid carrying γ, β and *exo* genes under control of P_{araB} promoter. Temperature sensitive replication. AmpR	(Datsenko and Wanner, 2000)
pBAD*Myc*-HisA	Expression vector. pBR322 derivative, *ara*BAD promoter (P_{BAD}), C-terminal *Myc* epitope tag, C-terminal 6xHis tag, *rrnB* transcription termination region, *araC*, ampR	Invitrogen™
pUT18C	BTH vector, p*lac*, N-terminal T18 fragment, AmpR	Euromedex™
pKT25	BTH vector, p*lac*, C-terminal T25 fragment, KanR	Euromedex™
pET-Duet-1	Expression vector. pBR322 derivative, ColE1 replicon, carrying two multiple cloning sites, each preceded by a T7*lac* promoter, *lacI*, ampR	Novagen ™
His-tagged protein constructs		
pSTM12506xHis	*STM1250* gene in L-arabinose inducible pBAD *Myc*HisA expression vector in frame with C-terminal 6xHis epitope, AmpR	GenScript
Overexpression constructs		
pSTM1250	*STM1250* gene in pBAD*Myc*-HisA expression vector without 6xHis tag, AmpR	This study
pDuet-STM1250	*STM1250* gene in IPTG inducible T7 promoter-1 pETDuet-1 expression vector, AmpR	This study

Plasmid	Description	Source
pDuet-STM1250-agsA	*STM1250* gene in IPTG inducible T7 promoter-1 and *agsA* gene in IPTG inducible T7 promoter-2 pETDuet-1 expression vector, AmpR	This study
Bacterial two hybrid constructs		
pUT18C-zip	BTH positive control	Euromedex™
pKT25-zip	BTH positive control	Euromedex™
pUT18C-*ibpA*	*ibpA* gene in pUT18CTH plasmid with N-terminal T18 fragment, AmpR	This study
pUT18C-*ibpB*	*ibpB* gene in pUT18C BTH plasmid with N-terminal T18 fragment, AmpR	This study
pUT18C-*STM1250*	*STM1250* gene in pUT18C BTH plasmid with N-terminal T18 fragment, AmpR	This study
pUT18C-*agsA*	*agsA* gene in pUT18C BTH plasmid with N-terminal T18 fragment, AmpR	This study
pUT18-*ibpA*	*ibpA* gene in pUT18 BTH plasmid with C-terminal T18 fragment, AmpR	This study
pUT18-*ibpB*	*ibpB* gene in pUT18 BTH plasmid with C-terminal T18 fragment, AmpR	This study
pUT18-*STM1250*	*STM1250* gene in pUT18 BTH plasmid with C-terminal T18 fragment, AmpR	This study
pUT18-*agsA*	*agsA* gene in pUT18 BTH plasmid with C-terminal T18 fragment, AmpR	This study
pKT25-*ibpA*	*ibpA* gene in pKT25 BTH plasmid with N-terminal T25 fragment, AmpR	This study
pKT25-*ibpB*	*ibpB* gene in pKT25 BTH plasmid with N-terminal T25 fragment, AmpR	This study
pKT25-*STM1250*	*STM1250* gene in pKT25 BTH plasmid with N-terminal T25 fragment, AmpR	This study
pKT25-*agsA*	*agsA* gene in pKT25 BTH plasmid with N-terminal T25 fragment, AmpR	This study

2.2 General laboratory techniques

2.2.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using Biomix Red (Bioline) or Phusion Green Hot Start II High-Fidelity PCR mastermix (Thermo Scientific). PCR compositions are shown in Table 3 for each polymerase. DNA template was either purified plasmid DNA (2.2.3) or for colony PCR a bacterial boilate preparation was used (2.2.1.1).

PCR was performed using a Techne^{TM} Prime Elite Thermal Cycler and the programmes used are detailed in Table 4.

2.2.1.1 Colony PCR

To prepare the bacterial DNA, a single colony was resuspended in 100 µL dH_2O and heated at 100 °C for 5 mins ("boilate"). The bacteria was then centrifuged at 15, 000 *x g* for 2 mins and the supernatant used in the PCR reaction (Table 3).

Table 3. PCR composition for reactions using Taq or Phusion polymerases.

PCR reagent	**Volume (µL)**	
***Taq* PCR**		
Biomix Red (2X)	12.5	25
Forward primer (10 µM)	0.5	1
Reverse primer (10 µM)	0.5	1
DNA	X	X
Water	∑25	∑50
Phusion HF PCR		
Phusion Green Hot Start Mastermix (2X)	12.5	25
Forward primer (10 µM)	0.5	1
Reverse primer (10 µM)	0.5	1
DNA	X	X
Water	∑25	∑50

Table 4. PCR programmes used in this study.

PCR stage	*Taq* PCR		Phusion HF PCR	
	Temp (°C)	**Time**	**Temp (°C)**	**Time**
1. Initial denaturation	96	3 min	98	30 sec
2. Denaturation	95	30 sec	98	10 sec
3. Annealing	(see note[$])	30 sec	(see note[$])	30 sec
4. Elongation	72	30 sec/kb	72	30 sec/kb
5. Repeat stages 2-4				
6. Final elongation	72	4 min	72	5-10
7. Hold (if required)	16	∞	16	∞

[$] annealing temperature is dependent on primer pair and requires optimisation for each reaction. Typically ~ 5 °C lower than primer melting temperature.

2.2.1.2 PCR product purification

Linear PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 30-50 µL dH_2O, preheated to 50 °C for increased DNA yield.

2.2.2 Gel electrophoresis

Agarose gel electrophoresis was used to analyse PCR products (2.2.1.2), purified plasmids (2.2.3) and digested plasmids (2.2.4). DNA was separated by size on a 1% (w/v) agarose gel containing 1X SYBR safe DNA gel stain (Invitrogen) in 1X TBE (Appendix B). DNA was mixed with 1X Gel loading dye (NEB) and run alongside the Hyperladder II (Bioline) size marker at 100 V for 45 mins, unless otherwise stated. DNA was visualised and imaged using a Molecular Imager® Gel Doc™ (BioRad).

2.2.2.1 Gel extraction

Digested plasmids (2.2.4) were purified by gel extraction, where required, to remove undigested vector. Digested products were separated by agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 20 µL dH_2O, preheated to 50 °C for increased DNA yield.

2.2.3 Plasmid purification

Plasmid DNA was extracted from 5 mL of bacterial O/N culture using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 50

µL dH_2O, preheated to 50 °C for increased DNA yield. DNA concentration and purity were analysed using a Nanodrop 2000c UV/Vis spectrophotometer (Thermo Scientific). DNA was stored at -20 °C.

2.2.4 Restriction digests

Genes of interest were cloned into the appropriate vector for a range of studies in this project: complementation studies (2.4), bacterial two hybrid screenings (2.10) and protein purification (2.12). In all cases, the gene of interest was amplified by PCR (2.2.1) using primers that resulted in the incorporation of a restriction site (Appendix C) at the 5' and 3' end of the PCR product. PCR products and plasmids were digested with the appropriate restriction enzyme for 1-2 hours at 37 °C before the enzyme was heat-inactivated at 65 °C for 20 mins. Restriction digest reaction compositions are presented in Table 5. Digested products were purified by PCR product purification (2.2.1.2) (gene products) or gel extraction (2.2.2.1) (vectors). Digested products were stored at -20 °C and used in ligation reactions (2.2.5).

Table 5. Restriction digest reaction compositions for digestion of insert (linear PCR product) and vector (plasmid) DNA.

Reaction component	Volume (µL)	
	Linear PCR product	**Plasmid DNA**
DNA	1 µg	3 µg
Enzyme A[$]	0.5	1.5
Enzyme B[$]	0.5	1.5
Reaction buffer	1	3
dH_2O	∑20	∑60

[$]See Appendix C for complete list of restriction enzymes used in this study.

2.2.5 Ligations

Ligation ratios were calculated (Equation 1) at a 3:1 ratio of insert:vector, using 100 ng vector DNA. Ligations of digested insert and vector DNA were performed O/N on ice using T4 DNA ligase (Promega). As a negative re-ligation control, ligations were also performed without insert DNA. Ligation reaction compositions are presented in Table 6. Following incubation, ligase enzymes were heat-inactivated at 70 °C for 10 mins.

$$\text{required mass of insert (ng)} = \frac{\{(\text{mass of vector in ng}) \times (\text{size of insert in kb})\}}{\text{size of vector in kb} \times \text{insert:vector ratio}}$$

Equation 1. Calculation of insert mass in a ligation reaction

Table 6. Ligation reaction compositions.

Reaction component	Volume	
	Insert and vector	**No insert control**
Vector	100 ng	100 ng
Insert	*3:1 ratio*	0
T4 DNA ligase reaction buffer (10X)	1	1
T4 DNA ligase	0.5	0.5
Water	∑10	∑10

2.2.6 Bacterial transformation

Bacteria were transformed with linear PCR product (2.2.1), purified plasmid DNA (2.2.3) or ligation reactions (2.2.5) by heat shock or electroporation, as appropriate.

2.2.6.1 Preparation of chemically competent cells

An O/N culture was used to inoculate 50 mL LB at a 1:100 dilution. Bacteria were grown at 37 °C, 200 rpm until an OD_{600} of 0.5 was reached. Cells were then transferred to pre-chilled 50 mL Falcon tubes and incubated on ice for 15 mins. The bacteria were pelleted by centrifugation at 4000 x *g*, 4 °C for 10 mins, the supernatant was removed and the pellet was resuspended in 20 mL ice-cold 0.1 M $CaCl_2$. Cells were incubated on ice for a further 15 mins before centrifugation as above. The pellet was resuspended in a final volume of 2 mL ice-cold 0.1 M $CaCl_2$. Competent cells were divided into 100 µL aliquots to be used immediately in heat shock transformation (2.2.6.3) or to be stored at -80 °C for future use. Cells to be stored were mixed in a final volume of 20 % (v/v) glycerol and snap frozen.

2.2.6.2 Preparation of electrocompetent cells

An O/N culture was used to inoculate 200 mL Lennox broth at a 1:100 dilution. Bacteria were grown at 37 °C, 200 rpm until an OD_{600} of 0.5 was achieved. Cells were then divided between pre-chilled 50 mL Falcon tubes and stored on ice for the remainder of the preparation. The bacteria were pelleted by centrifugation at 4000 x

g, 4 °C for 10 mins, the supernatant was removed and the pellet was washed by resuspension in 10 mL sterile ice-gold 10% glycerol, prepared in miili-Q water. The washing process was repeated for a total of three times. After the final centrifugation and removal of the supernatant, the pellet was resuspended in the residual 10% glycerol in the Falcon tube. Cells were then divided into 100 µL aliquots to be used immediately in electroporation or to be stored at -80 °C for future use.

2.2.6.3 Heat shock

Chemically competent cells (2.2.6.1) were kept on ice and mixed with 2 µL purified plasmid or 10 µL ligation reaction. The cells and DNA mixture were incubated on ice for 30 mins before a 45 sec incubation in a 42 °C water bath. Cells were transferred back to ice for 2 mins before 1 mL LB added and incubated at 37 °C for 1 h. Following recovery, a 100 µL aliquot of the transformation was plated onto LB containing the appropriate antibiotic. The remaining cells were pelleted, supernatant removed, pellet resuspended in 100 µL LB and plated as above. Plates were incubated statically at 37 °C O/N.

2.2.6.4 Electroporation

Electrocompetent cells were kept on ice and mixed with plasmid DNA (1-5 µL) or purified PCR product (10-20 µL for lambda red mutagenesis (2.3)). The cells and DNA mixture was transferred to an electroporation cuvette with a 0.2 mm gap (BioRad). Cells were pulsed with a single electrical pulse (EC2 setting on MicroPulser Electroporator (BioRad)). Following electroporation, 1 mL LB was added and cells were incubated at 37 °C with aeration at 220 rpm for 1-2 hours.

Following recovery, a 100 µL aliquot of the transformation was plated onto LB containing the appropriate antibiotic. The remaining cells were pelleted, supernatant removed, pellet resuspended in 100 µL LB and plated as above. Plates were incubated statically at 37 °C O/N.

2.3 Mutant Construction

The lambda red mutagenesis method described by Datsenko and Wanner (2000), in which a gene of interest is replaced with an antibiotic resistance cassette, was used to generate gene deletion mutants in this study (Figure 11). The method utilises the temperature sensitive pKD46 plasmid, encoding the bacteriophage lambda (λ) *gam*, *bet*, and *exo* genes for homologous recombination.

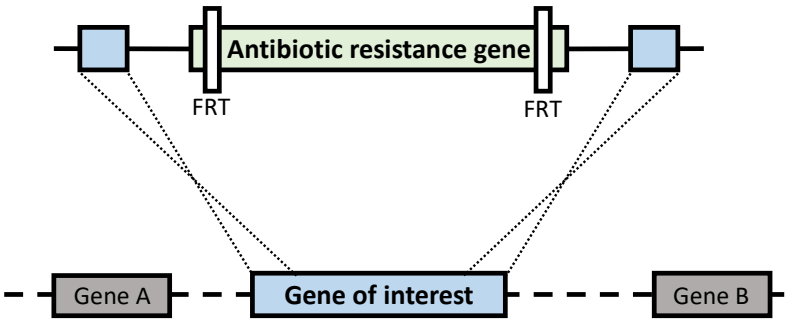
2.3.1 Preparation of FRT-flanked resistance cassette and transformation into SL1344 pKD46 competent cells for mutagenesis

A chloramphenicol (Cm) or kanamycin (Kan) resistance gene flanked by flippase (Flp) recognition target (FRT) sites was amplified by PCR from the plasmids pKD3 or pKD4, respectively. Primers were designed to incorporate 40 bp flanking regions of the gene of interest and 20 bp regions to amplify the appropriate resistance gene (Table 7) The resulting PCR product was purified and transformed by electroporation (2.2.6.2) into a host strain carrying the pKD46 lambda red recombination plasmid. For this, electrocompetent SL1344 pKD46 or MG1655 pKD46 cells were prepared (2.2.6.2) in Lennox broth, supplemented with 100 µg/mL ampicillin and 0.2% (w/v) L-arabinose to enable induction of the Red system. Single colonies present on LB agar following electroporation were screened by PCR using verification primers (Table 8) located ~100 bp upstream and downstream of the desired mutation and a reverse primer located within either the chloramphenicol or kanamycin resistance gene.

Successful *Salmonella* mutants were transduced into a clean WT SL1344 background by P22 transduction (2.3.2). Confirmed *E. coli* mutants were cured of the pKD46 temperature sensitive plasmid by 2 rounds of incubation at 43 °C and loss of pKD46 plasmid was verified with an inability to grow on LB agar containing 100 µg/mL ampicillin. This was followed by a final PCR verification to confirm successful mutagenesis.

1. PCR amplification of FRT-flanked antibiotic resistance gene with mutagenesis primers (M1 and M2).

2. Transformation of PCR product and induction of lambda red recombinase system for homologous recombination of antibiotic resistance gene and gene of interest.



3. Selection of antibiotic resistant transformants and PCR verification of mutagenesis with V1 and V2 primers.

Figure 11. Schematic representation of the lambda red recombination procedure for the generation of gene deletions in *E. coli* and *Salmonella*.

Table 7. Mutagenesis primers used in this study.

Primer Name	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
***Salmonella* Typhimurium mutants**		
ibpAB	ATGATGGAAGGTCTGACATTCTCGCTGATTTCAGGA GTTTGTGTAGGCTGGAGCTGCTTC	AAAGCCCCGCCATCTCTGGCGGGGCAAGGCAA GGAGCTCACATATGAATATCCTCCTTAG
STM1250-agsA	ATAAGCCAGTTTTTTGTTTCAGCGGTGACTGAAACAT ATTGTGTAGGCTGGAGCTGCTTC	GACAATGAAGGCCCGTCTAAACGGGCCTCCATT AACGCGACATATGAATATCCTCCTTAG
ibpA	ATGATGGAAGGTCTGACATTCTCGCTGATTTCAGGA GTTTGTGTAGGCTGGAGCTGCTTC	GAGCATGGGATGACAGGCCGCGCAACGCGACC CGACGGAACATATGAATATCCTCCTTAG
ibpB	TGTGAAGGTAAAAACTCGCTTCTTAGAAGGAGAAAT GATTGTGTAGGCTGGAGCTGCTTC	AAAGCCCCGCCATCTCTGGCGGGGCAAGGCAA GGAGCTCACATATGAATATCCTCCTTAG
STM1250	ATAAGCCAGTTTTTTGTTTCAGCGGTGACTGAAACAT ATTGTGTAGGCTGGAGCTGCTTC	TAGTCACGAATTTAAGTGGTTAACGAAAGTCTCA TCCCGTCATATGAATATCCTCCTTAG
agsA	GCCTTGATGTTGAACTTTTGAATAGTGATTCAGGAGG TTAGTGTAGGCTGGAGCTGCTTC	GACAATGAAGGCCCGTCTAAACGGGCCTCCATT AACGCGACATATGAATATCCTCCTTAG
phoP	TTAGCGCAATTCAAAAAGATATCCTTGTCCGCGTACG GTGGTGTAGGCTGGAGCTGCTTC	ATGATGCGCGTACTGGTTGTAGAGGATAATGCA TTATTACCATATGAATATCCTCCTTAG

Primer Name	Forward primer sequence (5’ – 3’)	Reverse primer sequence (5’ – 3’)
***Escherichia coli* mutants**		
ibpAB	TTAGCTATTTAACGCGGGACGTTCGCTGATAGCGAT ACGCGTGTAGGCTGGAGCTGCTTC	ATGCGTAACTTTGATTTATCCCCGCTTTACCGTT CTGCTACATATGAATATCCTCCTTAG

Table 8. Mutant verification primers used in this study.

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
***Salmonella* Typhimurium mutants**		
ibpAB	TTAACAATCAGTGCTGTCCC	ATATCACTCATCTTTTCTTC
ibpA	TTAACAATCAGTGCTGTCCC	TGGCTTTCGCCGCTGTTTTG
ibpB	CGGCGCAAACCTGGTCAACG	ATATCACTCATCTTTTCTTC
STM1250-agsA	AAATTCCAGTCAGTTGGCAA	GAATGTCGAGAGTGGACAAA
STM1250	AAATTCCAGTCAGTTGGCAA	TTATTCGCATCGCGCTTTTG
agsA	TGGCTGGTGGAGAAAAATTG	GAATGTCGAGAGTGGACAAA
STM1254	GCTGAGTCGCAAGGAACAGA	CGACATCAACAGGAGGGTGC
phoP	ATGCCATATGCCAAAGAAAG	GGGTGACTATTTGTCTGGTT
***Escherichia coli* mutants**		
ibpAB	CGCACATTATCACGGGAAAG	ATTTCCAGACCCATTTTTAC

2.3.2 *Salmonella* P22 transduction and selection for non-lysogens

Successful mutants generated by homologous recombination were transduced into a clean WT SL1344 background by P22 transduction. In the case of generation of the triple and quadruple mutants used in this study, Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* respectively, Δ*agsA* and Δ*STM1250*Δ*agsA* mutations were transduced into an SL1344 Δ*ibpAB* background.

2.3.2.1 Preparation of donor phage lysate

An O/N culture of the successful deletion mutant was prepared in 10 mL LB containing the appropriate antibiotic. This O/N was subsequently used in a 1:100 subculture in 10 mL LB and incubated at 37 °C, 220 rpm for 1 h. Following incubation, 20 µL P22 lysate was added and the culture was incubated at 37 °C, 220 rpm for 6 h. Cultures were mixed with 1 mL chloroform ($CHCl_3$) and incubated at 4 °C O/N. Chloroform was removed by centrifugation at 4000 x *g* for 20 mins. The supernatant (donor lysate) was removed and stored at 4 °C.

2.3.2.2 Transduction

An O/N culture of the appropriate recipient strain (2.3.2) was prepared. A 100 µL aliquot of O/N culture was added to 10 µL donor lysate and incubated at 37 °C for 45 mins. O/N culture only and donor lysate only controls were also prepared. Following incubation, cultures were plated onto LB agar plates containing the appropriate antibiotic and incubated O/N at 37 °C.

2.3.2.3 Selection for non-lysogens

Single colonies were streaked onto UCB indicator plates (green plates, Appendix A) to select for non-lysogenic colonies (Bochner, 1984). Green plates contain a high concentration of glucose in addition to pH indicators and enable the selection of P22 phage free colonies. As the *Salmonella* colonies grow they ferment the glucose, lowering the pH of the surrounding agar, and the colonies grow with a light green colour. Lysogenic colonies do not ferment glucose and produce dark coloured colonies. Light green colonies were selected, streaked onto LB agar containing the appropriate antibiotic and mutations confirmed by PCR as above (2.3.1) using the primers in Table 8.

2.4 Phenotype complementation

Genes of interest were cloned into the low copy expression vectors, pBAD*Myc*-HisA or pETDuet-1. In both cases, genes were cloned with a stop codon to prevent fusion

of the C-terminal epitope tag located downstream of the multiple cloning site in each of the vectors.

Genes were amplified by PCR using primers in Table 9 and purified by PCR product purification. The PCR products and the vector were digested with the appropriate restriction enzymes (Appendix C) and further purified by PCR product purification or gel extraction, respectively. Digested gene products and vectors were ligated O/N and transformed into chemically competent TOP10 cells. Single colonies were selected for screening; O/Ns were prepared of the single colonies and plasmids were purified. Purified vectors were sequenced (Eurofins) using sequencing primers Table 9.

For complementation, vectors were transformed by electroporation into the appropriate mutant background strain. For overexpression of genes cloned into the pBAD*myc*-HisA vector, cultures were supplemented with 0.2% (w/v) L-arabinose. For overexpression of genes cloned into the pETDuet-1 vectors, cultures were supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM.

Table 9. Cloning primers for complementation and overexpression studies.

Gene target and vector	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
pBAD*myc*-HisA		
STM1250	AAAAAACCATGGATTGTTCCTGGTGGTC	AAAAAAGCTTTCATTGCCATTTCCG
pETDuet-1		
STM1250	AAAAAACCATGGGTTTGTTCCTGGTGGTCTTACC	AAAAAAGAATTCTCATTGCCATTTCCGCTGTC
Sequencing primers		
pETDuet-1 P1	ATGCGTCCGGCGTAGAGGATC	CGATTATGCGGCCGTGTACAA

2.5 Stress susceptibility assays

A range of susceptibility assays were performed to assess the ability of mutant strains in this study to survive against different environmental stressors. In assays requiring the calculation of percentage survival, this was achieved by the calculation shown in Equation 2.

$$\% \text{ survival} = \frac{(\text{CFU/mL surviving cells})}{(\text{CFU/mL initially challenged cells})}$$

Equation 2. Calculation of percentage survival in susceptibility assays.

2.5.1 Growth curves

Susceptibility assay growth curves were conducted in a SpectraMax Plus 384 Microplate Reader (Molecular Devices). An O/N culture of bacteria was used to inoculate 1 mL media in Nunclon™ Delta Surface 24 well plates (Thermo Scientific) at a final OD_{600} of 0.1. Where stated, compounds of interest (1-6 mM H_2O_2 (Sigma), 1000-4000 U/mL bovine catalase (Sigma), 3 mM $CuCl_2$ (Sigma), 0.5 µM potassium tellurite (Sigma), 30 mM paraquat (methyl viologen, Sigma), 1 mM indole or 6% (w/v) sodium chloride (Sigma)) were added to each well. Plates were incubated at 37 °C and an OD_{600} reading was taken every hour, for up to 24 hours, with 3 sec of agitation prior to each reading. Assays were performed in biological triplicate with a minimum of two technical replicates to allow for statistical analysis.

2.5.2 Sensitivity spot plates

In order to test susceptibility of various mutant strains to compounds of interest, bacteria were spotted onto LB agar plates containing various compounds. LB agar plates were supplemented with the compound of interest (65 µg/mL vancomycin, 4 mM $CuCl_2$). Additionally, control LB agar plates without the compound of interest were prepared. O/N cultures of the mutant strains and the control SL1344 WT strain were standardised to an OD_{600} of 1.0 and 10-fold serially diluted in PBS to 1 x 10^{-7} CFU/mL. LB agar plates were spotted with 10 µL serially diluted bacteria, dried at room temperature and then incubated O/N at 37 °C. The following day, single colonies were counted the percentage survival was calculated for each strain by comparing the CFU/mL of treated and untreated bacteria (Equation 2). Sensitivity spot plates were conducted in biological triplicate with technical duplicates to allow for statistical analysis.

2.5.3 Heat shock

An O/N culture of the mutant strains of interest and the SL1344 WT control were used to inoculate 10 mL LB and were grown at 37 °C to OD_{600} 0.6 (mid-log phase). A t=0 mins sample was taken, serially diluted and spot-plated before the cultures were transferred to a water bath set to 50 °C. Bacteria were incubated at 50 °C for 4 h. At each time point, bacteria were 10-fold serially diluted and 10 µL aliquots spotted onto LB agar plates. Plates were dried at room temperature before being incubated O/N at 37 °C. Single colonies were counted after incubation and percentage survival calculated. Heat shock assays were performed in biological triplicate with technical duplicate to allow for statistical analysis.

2.5.4 Cold shock

An O/N culture of mutant strains of interest and the SL1344 WT control were used to inoculate 10 mL LB and were grown at 37 °C to OD_{600} 0.6 (mid-log phase). A t=0 mins sample was taken, serially diluted and spot plated before the cultures were incubated at 10 °C. Bacteria were incubated at 10 °C for 15 mins, 30 mins, 60 mins and 90 mins. At each time point, bacteria were 10-fold serially diluted and 10 µL aliquots spotted onto LB agar plates. Plates were dried at room temperature before being incubated O/N at 37 °C. Single colonies were counted after incubation and percentage survival at each time point was calculated. Cold shock assays were performed in biological triplicate with technical duplicate to allow for statistical analysis.

2.5.5 Antimicrobial killing assays

Antimicrobial challenge killing assays were performed according to Appia-Ayme et al. (2012) with minor alterations. An O/N culture of mutant strains of interest and the SL1344 WT control were used to inoculate 50 mL LB and grown at 37 °C to an OD_{600} of 0.1. An aliquot of culture was taken, 10-fold serially diluted and 10 µL spots plated onto LB agar to serve as a pre-challenged control. To the remaining culture, either polymyxin B or colistin was added, at the final concentration stated in the figure legends, and the bacteria were incubated at 37 °C, 220 rpm for 1 h. Following incubation, an aliquot was taken and serially diluted as above. Plates were dried at room temperature before incubation O/N at 37 °C. The percentage survival was calculated according to Equation 1. Antimicrobial killing assays were performed in biological triplicate with technical duplicates to allow for statistical analysis.

2.5.6 Acid tolerance assays

Acid tolerance response (ATR) assays were performed according to Lee et al. (2007) with modifications. WT SL1344 and mutant strains were grown O/N in 10 mL carbon-

free (NCE) medium (Appendix A) pH 7.0, supplemented with 0.4% (w/v) glucose and 400 µg/mL histidine.

For stationary phase adapted ATR assays, O/N cultures were standardised in NCE medium to an OD_{600} of 0.4 (6 x 10^8 CFU/mL). Standardised cultures were washed twice by centrifugation at 13,500 x *g* and resuspended in NCE adjusted to pH 4.4 with HCl or pH 6 with acetic acid, supplemented with 0.4% (w/v) glucose. Bacteria were incubated statically at 37 °C for 2 hours. Following incubation, cultures were washed once by centrifugation at 13,500 x *g* and resuspended in NCE adjusted to pH 3.0 with HCl or pH 4 with acetic acid, supplemented with 0.4% (w/v) glucose. Following the wash and resuspension, an aliquot of culture was taken, 10-fold serially diluted in NCE pH 7.0 and 10 µL spots plated onto LB agar to serve as a t=0 mins control. Bacteria were incubated statically in acidified NCE for 15, 30, 45 and 60 mins at 37 °C. At each time point, an aliquot of culture was serially diluted in NCE pH 7.0 and plated as for the t=0 mins sample. For phenotypic complementation, WT SL1344 WT and Δ*STM1250* mutant strain carrying the pETDuet-1 or pETDuet-STM1250 vectors were grown in the presence of IPTG during adaptation at pH 4.4 and acid shock at pH 3.0.

For ATR un-adapted assays, O/N cultures were standardised in NCE medium to an OD_{600} of 0.4 (6 x 10^8 CFU/mL). Standardised cultures were washed twice in NCE adjusted to pH 3.0 with HCl and a t=0 mins aliquot was taken and treated as above. Bacteria were then incubated statically at 37 °C for 30, 60 and 90 mins. At each time point, an aliquot was taken, 10-fold serially diluted in NCE pH 7.0 and 10 µL spots plated onto LB agar.

Under all conditions, percentage survival was calculated according to Equation 2. ATR assays were performed in biological triplicate with technical duplicate to allow for statistical analysis.

2.5.7 Minimum bactericidal concentration assays

Minimum bactericidal concentration (MBC) assays were performed in 96-well plates (Thermo Scientific) according to Clinical and Laboratory Standards Institute (CLSI) guidelines for determination of minimum inhibitory concentration (MIC) (CLSI, 2019). O/N cultures of WT SL1344 and mutant strains were standardised in LB to an OD_{600} of 0.08 and 100 µL standardised culture added to each well of the 96-well plate. Polymyxin B or colistin was added to a final concentration of either 16 µg/mL or 12 µg/mL to the first column of the plate and 2-fold serial dilutions were performed across the plate. Bacteria were incubated statically at 37 °C O/N (for at least 18 hours) before

the OD_{600} was measured. Assays were performed in biological triplicate with technical quadruplicate to allow for statistical analysis.

2.6 Tissue Culture

2.6.1 Cell lines and culture medium

The murine macrophage line RAW264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X non-essential amino acids (NEAA) and 2 mM L-glutamine (Sigma). Cells were maintained at 37 °C in a 5% (v/v) CO_2 atmosphere.

2.6.2 Long term storage and resurrection of cell lines

For long-term storage, cell lines were maintained in Recovery™ Cell Culture Freezing Medium (Gibco) in liquid nitrogen. For resurrection, cell culture vials were thawed in warm water and added to 5 mL supplemented DMEM. Cells were pelleted by centrifugation at 4000 x *g* at 20 °C for 5 mins to remove DMSO. Supernatant was discarded and cell pellet was resuspended in 7 mL supplemented DMEM (pre-warmed to 37 °C) and transferred to a 25 cm^3 (T25) tissue culture flask (Thermo Fisher). For propagation, RAW264.7 cells were passaged into 75 cm^3 (T75) flasks with a final volume of 21 mL.

2.6.2.1 RAW264.7 cell line maintenance

Cells were passaged into fresh T75 flasks and seeded on the same day into 24 well plates for experiments when at 80 – 100% confluency. RAW264.7 cells were used between passages 4 and 20. All reagents were pre-warmed to 37 °C prior to use.

RAW264.7 cells were passaged every 3 days. Spent media (10 mL) was removed and cells were detached from the flask surface using a cell scraper (Fisher Scientific) and thoroughly resuspended. For passaging, 3 mL resuspended cells were transferred to a new T75 flask containing 18 mL pre-warmed supplemented DMEM (passage ratio 1:3).

2.6.2.2 RAW264.7 cell seeding

For seeding, 30 µL resuspended cells were transferred to a haemocytometer. Cell concentration was determined by counting the number of cells in an average of three 4x4 regions of the haemocytometer, multiplied by 10^4. Cells were seeded into Nunclon™ Delta Surface 24 well plates (Thermo Fisher), in a volume of 1 mL, at a concentration of 1 x 10^6 cells/mL (RAW264.7). Prior to infection assays (2.7), seeded cells were incubated at 37 °C, 5% (v/v) CO_2 for 24 hours RAW264.7.

2.6.3 Frozen cell stock preparation

For cryopreservation, cells were grown in a T75 tissue culture flask to ~80% confluency. Cells were detached from the flask surface (as per 2.6.2.1) and resuspended in 10 mL supplemented DMEM. Cells were prepared for cryopreservation in Recovery™ Cell Culture Freezing Medium (Gibco) according to the manufacturer's instructions.

2.7 Infection assays

Mutant strains in this study were assessed for their ability to proliferate within RAW264.7 macrophage cells.

2.7.1 Macrophage intracellular survival assays (gentamicin protection assay)

Macrophages were seeded in 24 well plates 24 h prior to infection. For activation, 1000 U/mL IFN-γ was added 3 hours after seeding. Bacteria were freshly cultured on LB agar and used to prepare a bacterial dose of 10^7 cells/mL in supplemented DMEM. Macrophages were infected with 1 mL of the bacterial dose, resulting in a multiplicity of infection (MOI) or 10:1. Cells were incubated at 37 °C, 5% (v/v) CO_2. After 1 hour, the bacterial dose was removed, RAW264.7 cells were washed twice in 1x PBS, treated with 100 µg/mL gentamicin in DMEM for 1 hour before lysis or further incubation in 10 µg/mL gentamicin in DMEM. For inhibition of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, 250 µM apocynin (acetovanillone, 4-hydroxy-3-methoxyacetophenon, Sigma) was added with the bacterial dose, with each gentamicin dose and after 8 hours. For lysis, RAW264.7 cells were washed three times in 1X PBS and incubated in 1% (v/v) triton X-100 and 0.1% (w/v) SDS in PBS for 15 mins. Lysates were 10-fold serially diluted and 10 µL spots plated onto LB agar. Assays were performed in a minimum of biological duplicate with technical replicates to allow for statistical analysis.

2.8 Lipopolysaccharide analysis

2.8.1 Lipopolysaccharide extraction

LPS were extracted according to Morris et al. (2018). O/N cultures of WT SL1344 and mutant strains were standardised to an OD_{600} of 1.0 in 1 mL LB. Bacteria were pelleted by centrifugation at 18, 000 x *g* and resuspended in 100 µL LPS lysis buffer

(Appendix B). Cells were heated to 100 °C for 10 mins in lysis buffer, pelleted as before and the supernatant was transferred to a clean Eppendorf tube. To remove cellular proteins, lysate was incubated with 250 µg/mL proteinase K (NEB) at 60 °C for 1 hour. Proteinase K was inactivated with a 10 min incubation at 98 °C. LPS preparations were stored at -20 °C.

2.8.2 Silver staining

LPS preparations (10 µL) were mixed with 10 µL 2X Laemelli sample buffer (Sigma) and 20 µL run on a 4-20% Bis-Tris precast gels (Genscript) 25 mA for 2 hours. The gel was rinsed in milli-Q water and fixed O/N in 40% ethanol and 10% acetic acid in milli-Q water. LPS were stained using the SilverQuest silver staining kit (Invitrogen) according to the manufacturer's instructions. Gel was imaged on a Molecular Imager® Gel Doc™ (BioRad).

2.9 Quantitative real-time polymerase chain reaction (qRT-PCR)

2.9.1 RNA extraction

2.9.1.1 Cell harvesting and mRNA stabilisation

Cells were harvested for gene expression analysis under polymyxin B stress. For polymyxin B stressed cells, bacteria were treated according to (Navarre et al., 2005). LB (50 mL) was inoculated 1:100 with O/N culture and grown at 37 °C to an OD_{600} of 0.2. Polymyxin B was added at a final concentration of 1 µg/mL. Bacteria were grown for 40 minutes with polymyxin B before a 4 OD unit aliquot of cells were incubated on ice for 30 mins. Cells were harvested by centrifugation at 4000 rpm 4 °C for 10 minutes. The supernatant was removed and cell pellets were stored at -80 °C.

2.9.1.2 RNA extraction and analysis

Cell pellets (2.9.1.1) were defrosted on ice and resuspended in 200 µL 1X Tris-EDTA buffer (Sigma) containing 50 µg/mL lysozyme. Cell resuspension was halved and RNA extracted in parallel using the SV Total RNA Isolation System (Promega) kit. For preparation of cleared lysate, the cell suspension was mixed with 75 µL dilution buffer followed by 350 µL RNA dilution buffer and heated at 70 °C for 3 mins. The lysate was cleared by a 10 min centrifugation at 12000 x *g* and the cleared lysate transferred to a new RNase free tube. RNA was extracted according to the spin purification protocol for SV Total RNA Isolation System (Promega). Duplicate preparations were

each eluted in 50 µL sigma water and pooled. Total RNAs were treated with TURBO DNA-*free*™ (Invitrogen) according to the manufacturer's instructions.

RNA quality was analysed on a 1.5% (w/v) agarose gel in 1X TBE prepared with Sigma water. RNA was diluted 1:10 in Sigma water and mixed with 1X loading dye. Agarose gels were run at 100 V for 25 mins. RNA quantity was analysed by determination of the 260/230 and 260/280 ratios using a Nanodrop 2000c UV/Vis spectrophotometer (Thermo Scientific). RNA was snap frozen in aliquots and stored at -80 °C.

2.9.2 cDNA synthesis

Total RNA (2 µg) was used for cDNA synthesis and was reverse transcribed using 250 ng random primers (Invitrogen) and SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was stored at -20 °C.

2.9.3 qRT-PCR primer design and optimisation

Gene specific qRT-PCR primers Table 10 were designed using Primer3web v4.1.0 (http://primer3.ut.ee/) and the following criteria:

- product size range of 150 – 200 bp
- 50 - 55% GC content
- melting temperature (Tm) range of 62 - 68 °C
- maximum Tm difference of 5.0 °C.

Primers were assessed for their ability to generate a single product in a qPCR reaction by melt curve and agarose gel electrophoresis analysis. In addition, primer efficiency was determined by standard curve analysis using serial dilutions of SL1344 chromosomal DNA.

Appropriate primer concentration was determined using SL1344 chromosomal DNA. Two concentrations were tested, 250 nM and 500 nM and melt curves analysed to determine the optimal concentration. Primer optimisation qPCR reactions were performed in 96-well PCR plates (BioRad) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) with a final reaction volume of 10 µL.

Table 10. qRT-PCR primers used in this study.

Gene target	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	Source
STM1250	GCGCTGGGTTTGATAATGGA	TTCTCCACCAGCCAAATTGA	This study
pagC	GGCACGGTAAAGGCGACATT	ATTCATCTGTACACCCGCGC	This study
ampD	ATGACGAAAAACCGTCCTTG	GGATCTATCGTTCCGGTGAA	(Appia-Ayme et al., 2012)
gyrB	GCGTGAACTGTCATTCCTGA	TACCGTCTTTTTCGGTGGAG	(Deekshit et al., 2015)

2.9.4 qRT-PCR

qRT-PCR reactions were performed in 96-well PCR plates (BioRad) sealed with MicroSeal film (BioRad) to prevent evaporation of reaction components. Each well contained 10 µL SYBR ® Green JumpStart ™ *Taq* ReadyMix™ (Sigma), 1 µL cDNA, 1 µL forward primer (500 nM), 1 µL reverse primer (500 nM), 7 µL RNase free dH_2O (Sigma), resulting in a final reaction volume of 15 µL. Following preparation, plates were centrifuged at 1000 x *g* for 1 min to ensure that reagents were collected at the bottom of the wells. Each reaction was performed in technical duplicate, alongside no reverse transcriptase and no template controls, with three biological repeats for each condition. qRT-PCR reactions were performed according to (Table 11) using the BioRad CFX96™ thermo-cycler.

Table 11. qRT-PCR programme used in this study.

PCR stage	Temperature (°C)	Time
1) Initial Denaturation	94	3 mins
2) Denaturation	94	15 secs
3) Annealing and Elongation	50	1 min
4) Repeat stages 2 and 3	x 39	

2.9.4.1 The ΔΔCt method for relative quantification of gene expression

mRNA fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The C_T values for the non-treated (polymyxin B free) and treated (1 ug/mL polymyxin B) samples of the genes of interest (*pagC* and *STM1250*) were normalised to *gyrB* and *ampD* housekeeping controls (ΔC_T). To determine $\Delta\Delta C_T$, the ΔC_T of the non-treated samples was subtracted from the ΔC_T of the corresponding treated sample. Fold-change in expression was determined by calculating $2^{-\Delta\Delta Ct}$.

2.10 Bacterial two-hybrid assays

The bacterial-two hybrid (BTH) assay, originally described by Karimova et al. (1998), enables the detection and characterisation of protein-protein interactions within an *E. coli* host strain (BTH101). The assay utilises two specifically designed vectors, pKT25 and pUT18C, each of which carry two fragments of the catalytic domain of the *cya* gene, T25 and T18 respectively, encoding the *Bordetella pertussis* adenylate cyclase.

Single genes of interest, of which protein-protein interactions are to be tested, are cloned into the vectors which are subsequently co-transformed into the BTH101 host strain, deficient in endogenous adenylate cyclase activity. Only if the proteins of interest interact *in vivo* will the T25 and T18 catalytic fragments come together and adenylate cyclase activity restored. Protein-protein interactions are screened in the presence of x-gal, enabling blue-white screening.

2.10.1 Vector construction

Genes of interest were amplified by high-fidelity phusion PCR (2.2.1) using primers incorporating restriction sites to generate a linear PCR product with an EcoRI and XbaI at the 5' and 3' end respectively. Digested PCR products were ligated into pUT18C or pKT25 bacterial two hybrid vectors (Table 12). Ligations were transformed by heat shock into TOP10 and the presence of the incorporated gene confirmed by colony PCR and sequencing.

Table 12. Primer sequences used in this study for bacterial two hybrid vector construction and sequencing.

Gene name	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
ibpA	AAAAAATCTAGAAATGCGTAACTTTGATTT	AAAAAAGAATTCTCAGTTAATTTCGATACG
ibpB	AAAAAATCTAGAAATGCGTAACTACGAT	AAAAAAGAATTCTTAGCTATTTAATGCGGA
STM1250	AAAAAATCTAGAATTGTTCCTGGTGGTC	AAAAAAGAATTCTCATTGCCATTTCCGCTG
agsA	AAAAAATCTAGAAATGGCACTCAGAACC	AAAAAAGAATTCTTATGATTTGTGTTCAAT
Sequencing primers		
pKT25	GATTCGGTGACCGATTACC	GCCAGGGTTTTCCCAGTCAC
pUT18C	GTCGCTGGGCGCAGTGGAACG	AGAGCAGATTGTACTGAGAG

2.10.2 Bacterial two-hybrid assay

Cloned BTH vectors were co-transformed into *E. coli* BTH101 by heat shock. Following recovery, bacteria were plated onto LB agar containing 50 µg/mL kanamycin and 100 µg/mL ampicillin. For the BTH assay, five single colonies per transformation were patched onto LB agar containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, 0.5 mM IPTG and 40 µg/mL x-gal. Plates were incubated at 30 °C and photographed every 24 hours for 3 consecutive days.

2.11 β-galactosidase activity assays

β-galactosidase activity assays were utilised in this study to quantify protein-protein interactions observed during bacterial two-hybrid assays (2.10).

O/N cultures of BTH101 strains co-transformed with pKT25 and pUT18C vectors for BTH assays (2.10), encoding proteins of interest, were used to sub-culture 10 mL LB containing kanamycin, ampicillin, IPTG at a 1:100 dilution and incubated at 30 °C. Cells were grown to an OD_{600} of 0.6 (mid-log phase) before a 100 µL aliquot was taken and incubated on ice for 20 mins. Cells were permeabilised with one pasteur pipette drop of 1% (w/v) SDS and two pasteur pipette drops of chloroform. The cell suspension was vortexed briefly and incubated at 30 °C for 5 mins.

β-galactosidase assays were performed in a 96 well plate. Assay buffe (Z-buffer, Appendix B)r was aliquoted in 90 µL volumes per well and 10 µL permeabilised cell suspension was added. The reaction substrate ONPG was freshly prepared in 0.1 M phosphate buffer (Appendix B), 25 µL was added to each well and the plate was incubated at 30 °C. The incubation period was timed and once o-nitrophenol production had begun and the solution colour had begun to yellow, 400 µL of 1M Na_2CO_3 was added to stop the reaction. The OD_{420} and OD_{550} of the solutions was immediately recorded on a Spectramax M5 spectrophotometer (Molecular Devices). β-galactosidase activity was determined in Miller units (Miller, 1972) (Equation 3).

$$\text{Miller units} = 1000 \times \frac{A420-(1.75*A550)}{A600 \times v \times t}$$

Equation 3. Calculation of Miller units where t is equal to the time of the reaction in minutes and v is equal to the volume of culture used in the assay in mL (Miller, 1972).

2.12 Protein overexpression and purification

The *STM1250* gene was cloned (GenScript) into the pBAD*myc*-HisA expression vector for the addition of a C-terminal 6xHis-tag epitope, under the control of the L-arabinose inducible *ara*BAD promoter.

2.12.1 Overexpression trials

Small scale overexpression trials were performed with a culture volume of 50 mL. O/N cultures, supplemented with ampicillin, of protein expression strains carrying either the pSTM12506xHis overexpression construct or the *pBADMyc*-HisA empty vector (Table 2) were used at a 1:100 dilution to inoculate 50 mL Lennox broth, supplemented with 100 µg/mL ampicillin. *E. coli* TOP10 cultures were grown to an OD_{600} of 0.6, at which point a 1 mL non-induced sample was taken. The sample was pelleted by centrifugation (2 min, 14, 800 x *g*), snap frozen and stored at -20 °C. To the remainder of the culture, 0.2% (w/v) arabinose was added and incubated at 16 – 37 °C for 4 hours – O/N. Following incubation, 1 mL samples of induced culture were taken and harvested and stored as above for non-induced samples.

2.12.1.1 Expression trial SDS-PAGE analysis

Protein overexpression was analysed by SDS-PAGE. Cell pellets were defrosted on ice and resuspended in 50 µL laemelli sample buffer (Sigma) before boiling at 100 °C for 5 mins. Cell debris was removed by centrifugation and 10 µL of the supernatant was loaded onto a 4-20% SDS-PAGE precast gel (Sigma) alongside Pageladder (BioRad). Gels were run in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (BioRad) at 100 V, 80 mA for 1 hour in 1X TruPAGE (Sigma) running buffer. Gels were stained with Instant Blue gel stain (Expedeon) and imaged using a Molecular Imager® Gel Doc™ (BioRad).

2.12.1.2 Western blot

Protein overexpression was analysed by western blot to confirm the presence of the his-tag. Samples were prepared and SDS-PAGE gels were run as described above. Following electrophoresis, the gel was rinsed in dH_2O and incubated in 20% ethanol for 15 mins with gentle agitation. Proteins were transferred to a nitrocellulose membrane (iBlot). The membrane was blocked in 5% (w/v) fat-free skimmed milk powder in 1X TBST for 1 hour at room temperature with gentle agitation. Following blocking, the membrane was incubated in 20 mL of a 1:20,000 dilution of an anti-His HRP conjugated antibody (Qiagen) in blocking solution O/N at 4 °C. The membrane was washed 3 times in 1X TBST, 1 min per wash, with gentle agitation. The membrane was developed and His-tagged protein detected by chemiluminescence

using the Pierce™ ECL Western Blotting Substrate kit, according to the manufacturer's instructions (Thermo Scientific).

2.12.2 Large-scale protein expression and cell harvest

Large scale cultures were grown in 1 L volumes containing 100 mg/mL ampicillin and 0.2% (w/v) L-arabinose. To harvest cells, cultures were centrifuged in 95 x 191 mm 1L volume polycarbonate centrifugation bottles (Beckman Coulter) in a Beckman Coulter Avanti® J-20 high performance centrifuge at 3450 x *g* (6000 rpm) using a JLA-8.1000 rotor (Beckman Coulter). Cell pellets were resuspended in 10 mL PBS, transferred to 50 mL Falcon tubes, snap frozen and stored at -20 °C.

2.12.3 Preparation of cell lysates

Cell pellets from 4 x 1L cultures, obtained in 2.12.2, were defrosted on ice with 50 mg lysozyme (from chicken egg white, Sigma Aldrich), 40 µg DNase I (from bovine pancreas, Sigma Aldrich) and one cOmplete mini EDTA-free protease inhibitor cocktail tablet (Roche) was added. Cells were lysed by three passes through an IEC French Press Cell Disrupter (Thermo Scientific) at 1000 psi. To prepare the cleared cell lysate and separate soluble proteins from cell debris, cell lysate was centrifuged for 1 hour at 205,000 x *g* (42,000 rpm) in an Optima XL100K ultracentrifuge (Beckman) using the Ti45 rotor (Beckman). The pellet was discarded and the supernatant was stored on ice until purification (2.12.4).

2.12.4 Purification of STM12506xHis

The STM1250 protein was purified on an ÄKTA Pure FPLC (GE Healthcare) using the HisTrap™cartridge. Prior to sample loading, the cartridge was equilibrated with 1 column volume (CV) of purification loading buffer (Appendix B) at a flow rate of 2 mL/min. The cell lysate supernatant (2.12.3) was applied to the column at a flow rate of 1.5 mL/min. Following sample application, the column was washed with 2 CVs wash buffer containing 20 mM imidazole (Appendix B) and the wash flow-through was collected in 50 mL Falcon tubes for SDS-PAGE analysis. Protein was eluted over a linear gradient of 0-100% of elution buffer containing 250 mM imidazole at a flow rate of 2 mL/min. Elution fractions were collected in 1 mL volumes for SDS-PAGE and western blot analysis.

2.12.5 Size-exclusion chromatography

Selected fractions were pooled and further purified by gel filtration size-exclusion chromatography on an ÄKTA Pure FPLC (GE Healthcare) using a Superdex 200 gel

filtration column (GE Life Sciences). Fractions were eluted in 2 mL volumes and analysed for protein content by SDS-PAGE and western blotting.

2.13 Data presentation and statistical analysis

Data was presented and analysed using Graphpad Prism software v 8.0. Experiments were performed in biological triplicate with a minimum of 2 technical replicates per experiment. Data were analysed by *t*-test or ANOVA, as specified in the figure legend.

Chapter 3 The contribution of IbpA, IbpB, AgsA and STM1250 to the protection of Salmonella against extracytoplasmic stress

Work in this chapter contributed to the following article:

Hews, C. L., Pritchard, E. J. and Rowley, G., 2019. The *Salmonella* Specific, σ^E-Regulated, STM1250 and AgsA, Function With the sHsps IbpA and IbpB, to Counter Oxidative Stress and Survive Macrophage Killing. *Frontiers in cellular and infection microbiology*, 9(263).

3.1 Introduction

As described in Chapter 1, the σ^E response regulates numerous genes of unknown function, that are assumed to contribute to the envelope stress response. The critical nature of such stress response proteins can often mean that there is functional redundancy between them. As a result, single deletion mutants may not always show significant phenotypes under stress inducing conditions. To this end, deletion mutants of multiple genes with potential overlapping function, may provide a route for improving our understanding of stress response proteins with a currently unknown function.

This chapter describes the σ^E-regulated small heat shock proteins IbpA, IbpB, and AgsA and the putative stress response protein STM1250. The role of these proteins in the *Salmonella* response to various environmental stresses is explored. As outlined below, we hypothesise that functional overlap exists between these four proteins and multiple gene deletion mutant strains were made to address this hypothesis.

3.1.1 The bacterial heat shock response machinery

The ability of *Salmonella*, and indeed all enteric pathogens, to tolerate extreme changes in temperature is crucial to its ability to survive in multiple environments and hosts with varying body temperatures. Heat treatment is routinely used in the food production industry as a means of sterilisation; therefore, understanding bacterial resistance to heat stress is important in order to reduce foodborne pathogen transmission (Hsu-Ming et al., 2012). In addition to its role in the food production industry, heat is a defence mechanism employed by the host innate immune system. Infections are commonly accompanied by development of a fever – an increase in core body temperature with the overarching aim of eliminating invading pathogens. But, when human infections arise from the poultry reservoir where the body temperature is 42 °C, *Salmonella* may be pre-adapted to human body temperature and the fever associated with the innate immune response (Dawoud et al., 2017).

Heat stress can be bactericidal through the disruption of membrane composition (via an increase in fluidity), protein homeostasis and ribosome functions (Denich et al., 2003, Li and Gänzle, 2016). For example, heat treatment of *E. coli* at 55 °C leads to increased OM permeability and the release of LPS molecules which are an essential protective feature of the OM (Tsuchido et al., 1985).

Temperature upshift induces the bacterial heat shock response. The cellular response against heat-induced damage involves the activation and regulation of the heat shock response regulator σ^H. In addition to σ^H, since heat shock is detrimental to the OM and leads to the aggregation of misfolded proteins, the σ^E regulon is also induced (Rouvière et al., 1995). Activation of the σ^H and σ^E responses were described in Chapter 1. Together, their induction upregulates genes required for the repair of damaged proteins and DNA arising from the sudden changes in temperature. This response has been studied extensively in *E. coli* and the upregulation of at least 20 specific heat shock proteins and transcriptional regulators following a temperature change of 30 °C to 42 °C has been demonstrated (Arsène et al., 2000). These heat shock genes and regulators are conserved in *Salmonella* and include chaperones and small proteins (DnaK, ClpB, GroEL chaperonins, IbpA, IbpB), proteases (Lon, HtrA) and the σ^H and σ^E sigma factors themselves. In addition to this core set of widely conserved heat shock proteins and transcriptional regulators, expression of a vast number of additional genes have been shown to be altered during heat shock. In *Salmonella*, Pin et al. (2012) identified that 120 genes were upregulated following exposure to a temperature increase of 25 °C to 45 °C and 102 of these genes remained up-regulated 30 minutes after *Salmonella* was returned to 25 °C.

3.1.1.1 Small heat shock proteins

sHsps are a highly conserved group of proteins found across eukaryotes and prokaryotes. The primary role of sHsps is to prevent the aggregation of folded and unfolded proteins, catalysing the folding of the latter via additional chaperones including DnaK and GroEL. Typically, although exclusions do exist, sHsps have (I) a molecular weight between 12 and 42 kDa, (II) the ability to form multimeric structures (a feature crucial to their chaperone properties), (III) the ability to function as an ATP-independent chaperone and (IV) a conserved α-crystallin domain (Narberhaus, 2002). The sHsp nomenclature can be somewhat misleading. Firstly, heat-inducible proteins that do not contain the α-crystallin domain, for example GroES and GrpE, are not included in this group, despite their important cellular roles in survival against heat stress (Narberhaus, 2002). In fact, it has been argued that 'sHsps' containing the α-crystallin domain should be described as α-Hsps (de Jong et al., 1998, Narberhaus, 2002). Secondly, studies have continued to identify numerous roles for sHsps that are not limited to the survival of heat stress. Such roles include survival against oxidative stress (Kitagawa et al., 2000, Matuszewska et al., 2008), metal stress (Pérez et al., 2007, Matuszewska et al., 2008, Ezemaduka et al., 2018, Lv et al., 2019) and host-associated stresses encountered during infection (Goeser et al.,

2015). As such, when investigating the roles of poorly understood α-crystallin proteins, roles in stress resistance beyond thermal tolerance should not be overlooked.

α-crystallins are structural chaperone proteins located in the vertebrate eye lens, required to prevent protein aggregation and cataract formation (Brady et al., 1997). The α-crystallin domain is highly conserved across all kingdoms of life (Paul et al., 2016), although it should be noted that some organisms do lack α-crystallin proteins with bacterial examples including (but not limited to) *Campylobacter jejuni*, *Helicobacter pylori, Neisseria* and *Haemophilus influenzae* (Narberhaus, 2002). The α-crystallin domain within sHsps is approximately 90 residues in length and is typically preceded by an unconserved N-terminal region and followed by a short C-terminal region (Narberhaus, 2002). The domain contains a consensus motif at the 3' end, formed of the amino acid sequence AxxxnGvL, as shown in Figure 13. To date, the precise role of the α-crystallin domain within a heat shock protein is not completely clear, although it is known that this domain is important for interactions between subunits. In particular, Wotton et al. (1996) identified that the 3' consensus motif of the α-crystallin domain is required for *Saccharomyces cerevisiae* Hsp42p oligomerisation. Despite this, the α-crystallin domain cannot form oligomers when expressed alone and does not possess *in vitro* chaperone activity (Studer et al., 2002), pointing towards an, as of yet, unidentified role for the N- and C-terminal regions of α-crystallin proteins in oligomerisation.

In bacteria, α-crystallins are important molecular chaperones (Jakob et al., 1993) with examples including IbpA and IbpB (conserved among multiple species), AgsA (*Salmonella*), GspA (*Legionella pneumophila*), HspA (*Vibrio cholerae*), Hsp20 (*Acinetobacter baumannii*) and YocM, YdfT, CotM (*Bacillus subtilis*) (Narberhaus, 2002).

3.1.2 IbpA and IbpB

Ibp (inclusion body protein) A and B (IbpAB) are encoded by the *ibpA* and *ibpB* genes, separated by only 110 nt in *Salmonella* (). The proteins are α-crystallin sHsps but were initially identified in *E. coli* during overexpression of heterologous proteins, specifically human prorenin, renin and bovine insulin-like growth factor 2 (Allen et al., 1992). The induction of IbpA and IbpB was found to correlate with the overexpression of insoluble proteins, resulting in the formation of inclusion bodies (Allen et al., 1992). Coupled to their expression and regulation, later studies identified that IbpA/B not only bind to overexpressed heterologous proteins, but also to endogenous protein

aggregates arising as a result of heat shock (Laskowska et al., 1996). IbpA is conserved among multiple Gram-negative bacteria including *Shigella, Salmonella*, *Klebsiella, Citrobacter* and *Pseudomonas* whilst IbpB is less well conserved (Figure 13).

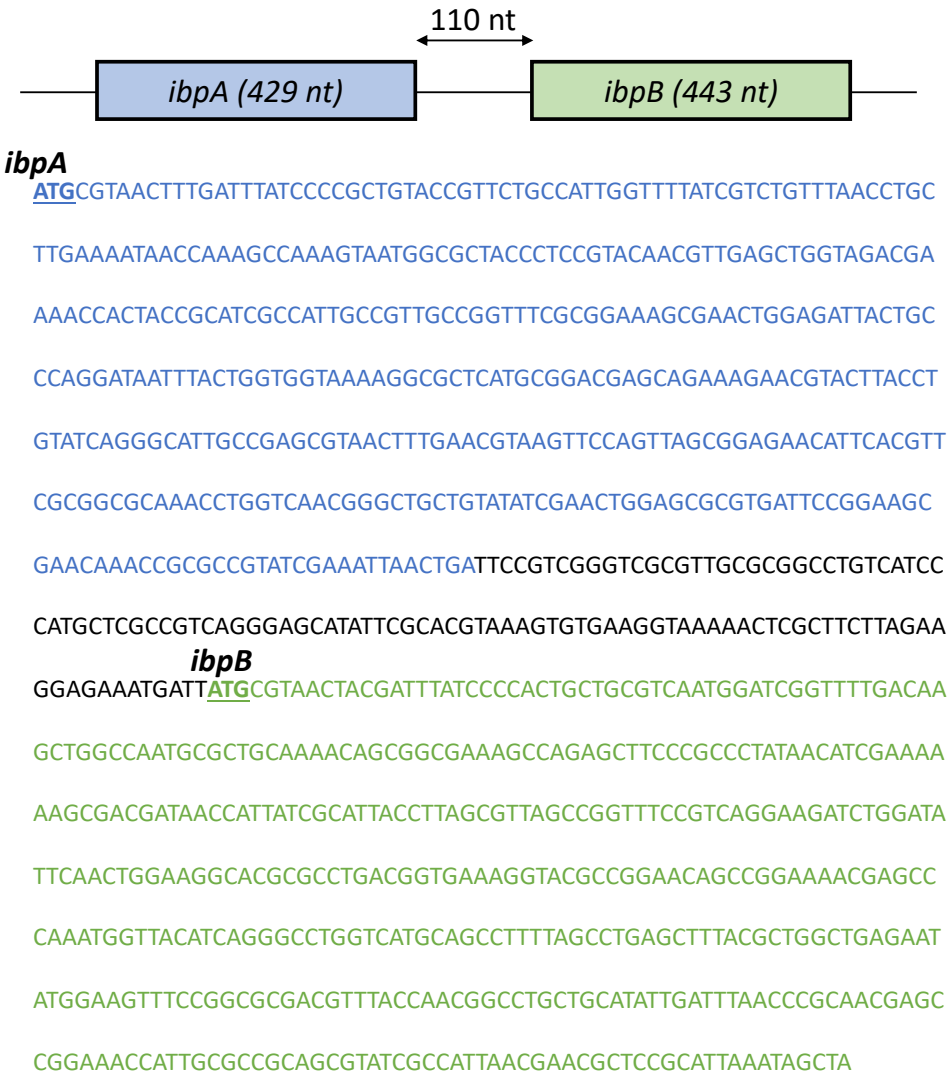


Figure 12. Genetic organisation of the *ibpA* and *ibpB* genes in *Salmonella* Typhimurium. The *ibpA* and *ibpB* genes are separated by a 110 nt region in *S.* Typhimurium.

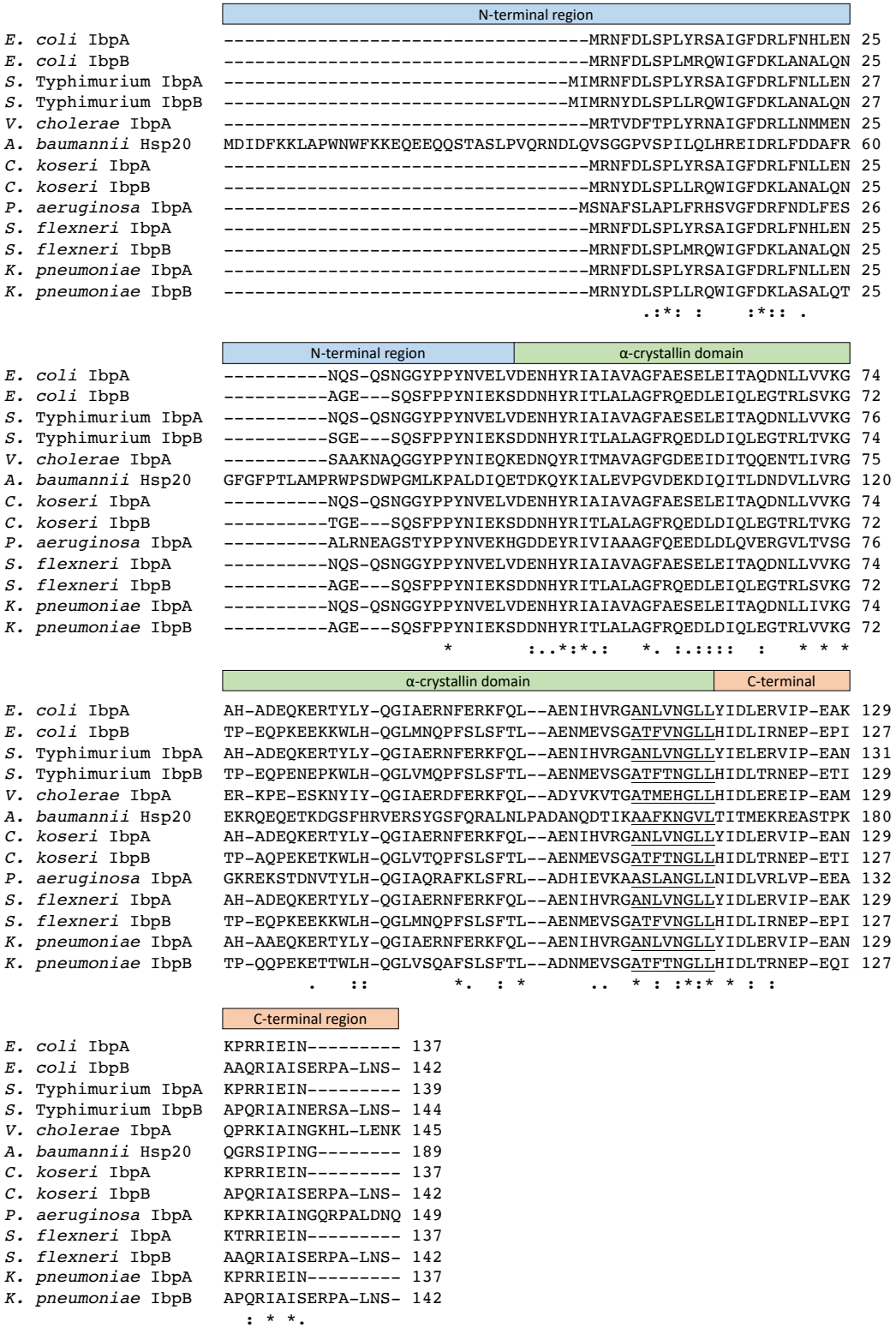


Figure 13. Amino acid alignment of IbpA and IbpB in Gram-negative bacteria. The N-terminal region, C-terminal region and the α-crystallin domain are indicated above the sequences. The consensus motif (AxxxnGvL) located at the 3' end of the α-crystallin domain is underlined in all sequences.

Figure 1 continued. Amino acid sequences were obtained from the NCBI database with accession numbers as follows: *Acinetobacter baumannii* Hsp20 (PST50138.1), *Pseudomonas aeruginosa* IbpA (NP_251816.1), *Vibrio cholerae* IbpA (AWB72831.1), *Escherichia coli* IbpA (NP_418142.1), *Salmonella* Typhimurium IbpA (CBW19866.1), *Citrobacter koseri* IbpA (QEU25751.1). Alignments were performed by Clustal Omega, asterisks (*) denote fully conserved residues, colons (:) denote conservation between residues with highly similar properties, full-stops (.) denote conservation between residues with weakly similar properties. Figure adapted from Narberhaus (2002).

ibpA/B are heat inducible (Chuang et al., 1993) and are regulated by the heat shock sigma factor σ^H, with *ibpA/B* expression reduced in *E. coli rpoH* mutants following heat treatment (Allen et al., 1992). However, Laskowska et al. (1996) still observed an increase in expression of IbpA and IbpB in an *E. coli* Δ*rpoH* mutant, indicating that additional regulatory mechanisms influence their expression. Later studies confirmed that indeed, *ibpA* and *ibpB* are also regulated by the extracytoplasmic sigma factor σ^E (Kuczynska-Wisnik et al., 2001).

In addition to transcriptional regulation by σ^H and σ^E, expression of *ibpA* is controlled at the post-transcriptional level by a 5' untranslated region (UTR) RNA thermometer of the ROSE (repression of heat shock gene expression) family (Waldminghaus et al., 2009). RNA thermometers (RNATs) are temperature responsive regions of mRNA that control gene expression (Kortmann and Narberhaus, 2012). At lower temperatures, RNATs fold into hairpin loops which block ribosomal access to the Shine-Dalgarno sequence, thereby preventing translation (Narberhaus et al., 2006, Kortmann and Narberhaus, 2012). At increased temperatures, the hairpin structures melt, releasing the Shine-Dalgarno sequence and enabling translation. The *ibpA* RNAT is predicted to form three separate hairpin loops (Waldminghaus et al., 2009). In particular, hairpin III has been shown to block ribosomal access to the Shine-Dalgarno sequence at temperatures below 35 °C (Waldminghaus et al., 2009). A putative RNAT has also been identified upstream of *ibpB*; *in vitro* studies have demonstrated the canonical mechanism of hairpin blocking ribosomal access to the Shine-Dalgarno sequence, but this particular control of *ibpB* gene expression has not been replicated during *in vivo* gene reporter fusion studies (Gaubig et al., 2011). Interestingly, the presence of *ibpA* was shown to influence the activity of the *ibpB* RNAT and therefore *ibpB* expression. These findings indicated cross-talk between the expression of the two sHsps (Gaubig et al., 2011). The dependence on the correct levels of expression of both IbpA and IbpB for their ability to function as molecular

chaperones is also indicated in multiple studies, identifying cross-talk and cooperation at the protein level: co-dependency is suggested due to the fact that purified IbpA forms fibrils in the absence of IbpB (Ratajczak et al., 2010). Despite this, further work will be required to determine precisely whether IbpA and IbpB must both be present for chaperone function. As indicated below (Table 14), *Vibrio cholerae* and *Pseudomonas aeruginosa* encode only the *ibpA* gene and there is no indication in the literature that a lack of IbpB in these bacteria leads to any deficiency in sHsp function.

In addition to cooperation with one another under stress, IbpA and IbpB have also been identified to function with other cytosolic chaperones involved in the heat shock response in *E. coli*, including DnaK, DnaJ and GrpE. Studies have indicated that IbpB is important in the stabilisation of proteins *in vitro*, as demonstrated using the heat-inactivated malate dehydrogenase (MDH) and urea-inactivated lactate dehydrogenase (LDH) model substrates (Veinger et al., 1998). Stabilisation prevents aggregation and these proteins are then delivered to the DnaK/DnaJ/GrpE chaperones for refolding (Veinger et al., 1998). Both IbpA and IbpB are required for efficient function of the Clp (Hsp100) and DnaK (Hsp70) stress response chaperones in the disaggregation of thermally aggregated proteins (Matuszewska et al., 2005, Ratajczak et al., 2009) with the group described as a functional triad (Mogk et al., 2003).

Being α-crystallin sHsps, IbpA and IbpB form high-order oligomeric structures as part of their chaperone functions. Current research does not suggest that the higher-order structures could be mixed IbpA/B hetero-oligomers although such structures have been observed for different sHsps in other bacterial species (Kuczyńska-Wiśnik et al., 2002). *In vitro,* purified IbpA and IbpB form large multimeric structures of between 2 and 3 MDa, which dissociate at high temperatures to smaller monomeric or oligomeric structures for IbpA and IbpB, respectively (Kitagawa et al., 2002). In addition, *in vitro* studies of IbpB have demonstrated the formation of 600 kDa oligomers. However, as indicated above, IbpA influences the function of IbpB and it is unclear as to whether structures of this size are formed *in vivo* (Shearstone and Baneyx, 1999).

Initial studies indicated that the cellular role of IbpAB was limited to the prevention of protein aggregation following heat shock. For example, overexpression of *ibpA* and *ibpB* provides *E. coli* tolerance to extreme temperatures. Despite this, survival of *E. coli* and *Salmonella* Δ*ibpAB* mutants are unaffected following heat shock at 50 °C (Thomas and Baneyx, 1998, Tomoyasu et al., 2003). Since the initial identification of IbpA and IbpB, multiple studies have investigated additional cellular roles of IbpA/B

and indicated that IbpA and IbpB are differentially regulated in response to stresses other that extreme heat shock. For example, IbpA and IbpB were shown to be of importance to *E. coli* cell division through interaction with the lipoprotein NlpI (Tao et al., 2015) and their roles in the protection against copper induced oxidative stress in *E. coli* has received attention (Matuszewska et al., 2008). Clearly an expansion of knowledge of their cellular role, especially in species beyond *E. coli*, is required. The roles of the IbpAB proteins in *Salmonella* are poorly studied in comparison to *E. coli*. Although protein function is often highly similar between these two species, key differences do exist. In this case, one such key difference is the presence of an additional sHsp, AgsA, of high similarity to IbpA and IbpB in the *Salmonella* genome. AgsA is not conserved in any other member of the *Enterobacteriaceae* family.

3.1.3 AgsA (STM1251)

AgsA (aggregation suppressing protein A) is an 18 kDa *Salmonella* specific protein containing an α-crystallin domain and, therefore in addition to IbpA and IbpB, is part of the *Salmonella* sHsp family. AgsA shares high sequence similarity to *Salmonella* IbpA and IbpB at 50% and 53.5% respectively, although sequence identity is rather low (29.5% for IbpA and 27.1% for IbpB). In addition to IbpA/B, AgsA shares 55% sequence similarity to the α-crystallin sHsp of *Legionella pneumophila*, GspA, although again, the percent identity between these proteins is relatively low at 35%.

AgsA was first identified in the thermally aggregated protein fraction of a *Salmonella dnaK52* mutant following incubation at 42 °C (Tomoyasu et al., 2003). When overexpressed in *E. coli* Δ*dnaK52* and Δ*rpoH* mutants, AgsA has been shown to increase survival against heat stress due to a reduction in thermally-aggregated proteins (Tomoyasu et al., 2003). Initial studies predicted that AgsA, like IbpA and IbpB, is able to prevent aggregation of non-native proteins during their overproduction and in addition, prevent heat-induced protein aggregation (Tomoyasu et al., 2003). Low level expression of AgsA is observed at 37 °C with protein levels significantly increased at 42 °C and 44 °C, corresponding to its role in the heat shock response (Tomoyasu et al., 2003). In addition to the reduction of protein aggregation *in vivo*, AgsA has also been shown to reduce aggregation of chemically and thermally denatured proteins *in vitro* (Tomoyasu et al., 2013).

agsA expression is regulated by multiple sigma factors; the housekeeping sigma factor RpoD, the heat shock sigma factor RpoH (Tomoyasu et al., 2003) and the extracytoplasmic sigma factor RpoE (Skovierova et al., 2006) which enables multiple levels of transcriptional control of *agsA* under numerous stress-inducing conditions.

In addition to transcriptional regulation, like *ibpA*, expression of *agsA* is environmentally controlled by a 5' UTR RNA thermometer. The *agsA* RNA thermometer is unique in that it was the first example of a FourU RNA thermometer (Kortmann and Narberhaus, 2012). Predicted to be comprised of two hairpin loops, the 57-nucleotide structure located upstream of *agsA* contains four consecutive uracil nucleotides that pair with the Shine-Dalgarno sequence, inhibiting ribosomal access. The RNA thermometer melts at temperatures exceeding 37 °C (Waldminghaus et al., 2007).

Structural analysis and X-ray crystallographic studies have identified that AgsA, similarly to IbpA and IbpB, forms oligomeric structures (Zhou et al., 2016). Additionally, AgsA forms unique fibril-like structural complexes which display chaperone activity (Shi et al., 2011) and an Ile-X-Ile motif located at the C-terminus of AgsA is important for oligomeric structure formation (Zhou et al., 2016).

Due to their sequence similarity, shared transcriptional regulators and the fact that AgsA is a member of the α-crystallin family, AgsA is predicted to functionally overlap with IbpAB. In addition, *agsA* forms an operon with a gene, *STM1250*, located 151 bp upstream (Skovierova et al., 2006).

3.1.4 STM1250

STM1250 is a 10 kDa putative cytoplasmic protein in *Salmonella*. Unlike IbpA, IbpB and AgsA, STM1250 is not a member of the α-crystallin containing sHsp family; however as indicated above, it forms an operon with *agsA* and is located only 151 bp upstream of the *agsA* gene () (Skovierova et al., 2006). Therefore, it is hypothesized that STM1250 functions in concert with AgsA and may therefore also cooperate with IbpAB. Despite lacking the characteristic sHsp α-crystallin domain, STM1250 has been linked to heat stress and is upregulated during recovery of *Salmonella* following incubation at 55 °C (Hsu-Ming et al., 2012).

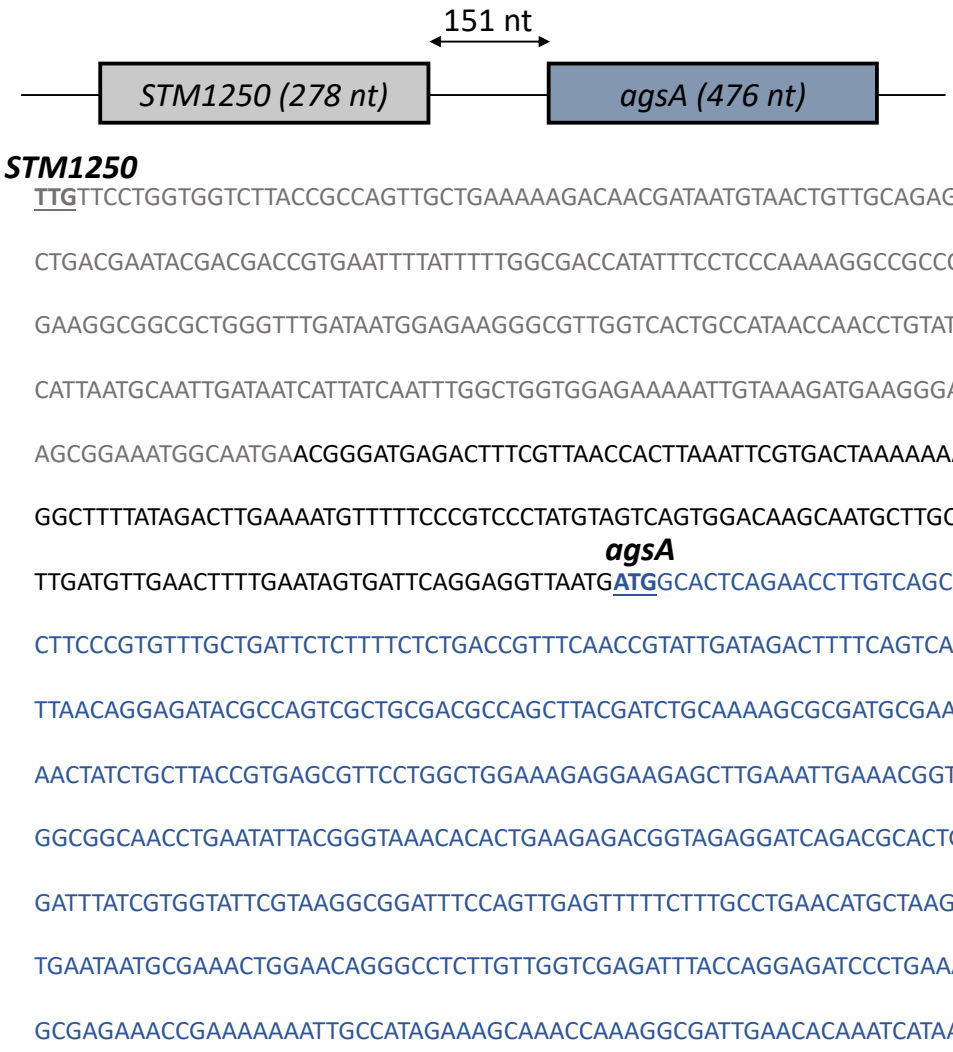


Figure 14. Genetic organisation of the *STM1250* and *agsA* genes in *Salmonella* Typhimurium. The *STM1250* and *agsA* genes are separated by a 151 nt region in *S.* Typhimurium.

Like AgsA, STM1250 is specific to *Salmonella*. The precise intracellular role of STM1250 is unknown and currently, STM1250 is annotated as a hypothetical protein. Interestingly, several regulatory studies point towards a potential role for STM1250 during infection. STM1250 is directly regulated by σ^E (Skovierova et al., 2006), a key regulator in the defence against heat and extracytoplasmic stress (Chapter 1). Furthermore, ChIP-seq analysis of the Fis regulon identified a Fis binding site located upstream of *STM1250* (Wang et al., 2013) and Monsieurs et al. (2005) also reported that a PhoPQ regulatory motif is located upstream of *STM1250* (Figure 15). Fis is a global regulator of transcription and controls the expression of *Salmonella* virulence genes, including members of the SPI-1 and SPI-2 regions (Wang et al., 2013). As

described in Chapter 1, PhoPQ is required for intracellular survival and resistance to antimicrobial peptides such as polymyxin B.

Interestingly, the STM1250 protein does not contain any conserved domains that would point towards its cellular role. As a result, the function of STM1250 is unknown, although its size and genomic proximity to *agsA*, in addition to its confirmed and predicted regulators indicates that it may function as a stress responsive protein in *Salmonella*.

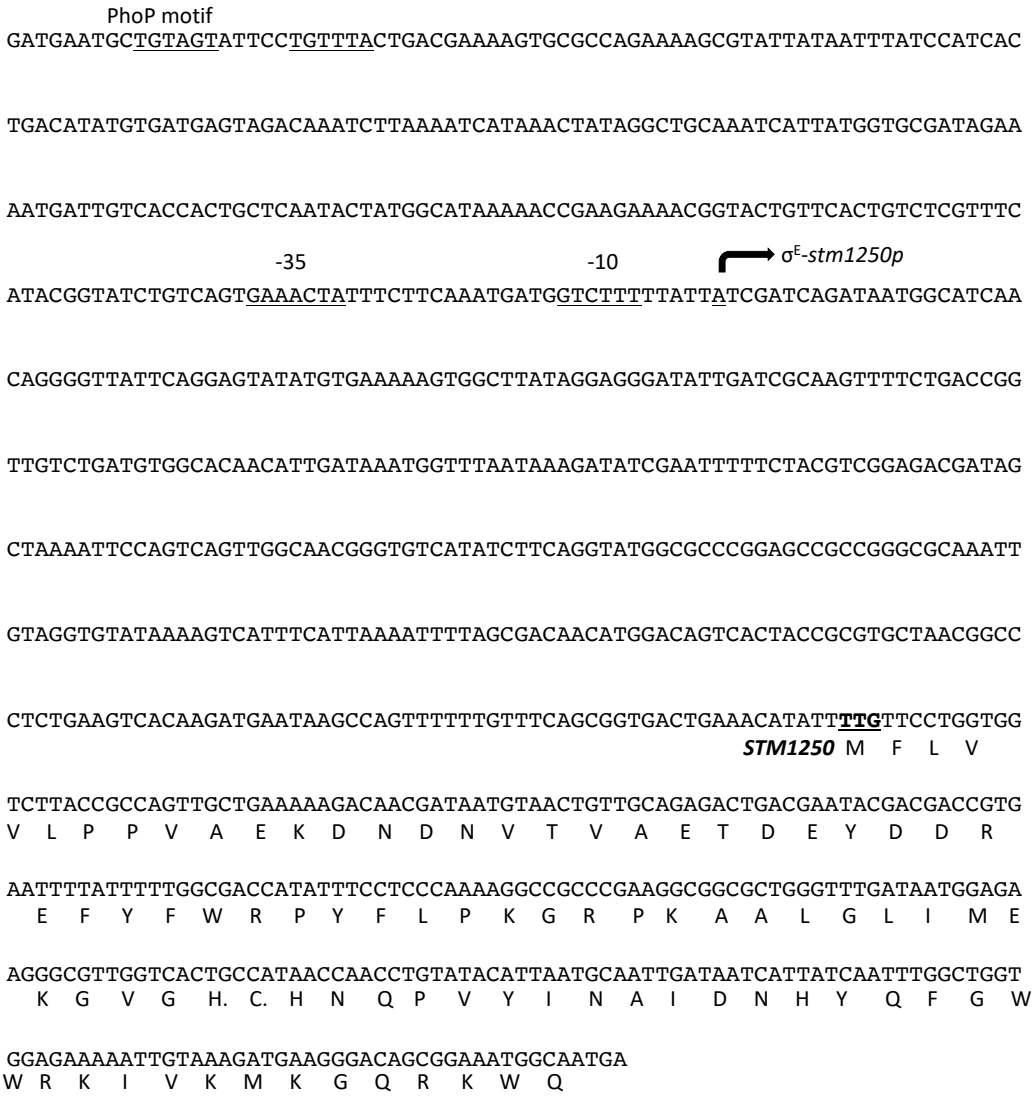


Figure 15. *STM1250* promoter and amino acid sequence. The proposed PhoP binding motif (Monsieurs et al., 2005) and the RpoE promoter (Skovierova et al., 2006) are both underlined.

3.1.5 sHsps, a role beyond heat tolerance?

Despite the sHsp nomenclature associated with IbpA, IbpB and AgsA, we hypothesise that these proteins function, in cooperation with STM1250, to protect *Salmonella* from extracytoplasmic stresses that are not limited to heat shock. In agreement with their hypothesised extended cellular roles, genome-wide RNA-seq studies have identified that all genes are differentially regulated under various stress-inducing conditions including osmotic stress and intracellular infection (Kröger et al., 2013, Canals et al., 2019) (Figure 16). These studies, coupled to the regulation of the four genes by the extracytoplasmic sigma factor, suggest that the proteins may contribute to envelope maintenance processes during exposure to different extracytoplasmic stresses.

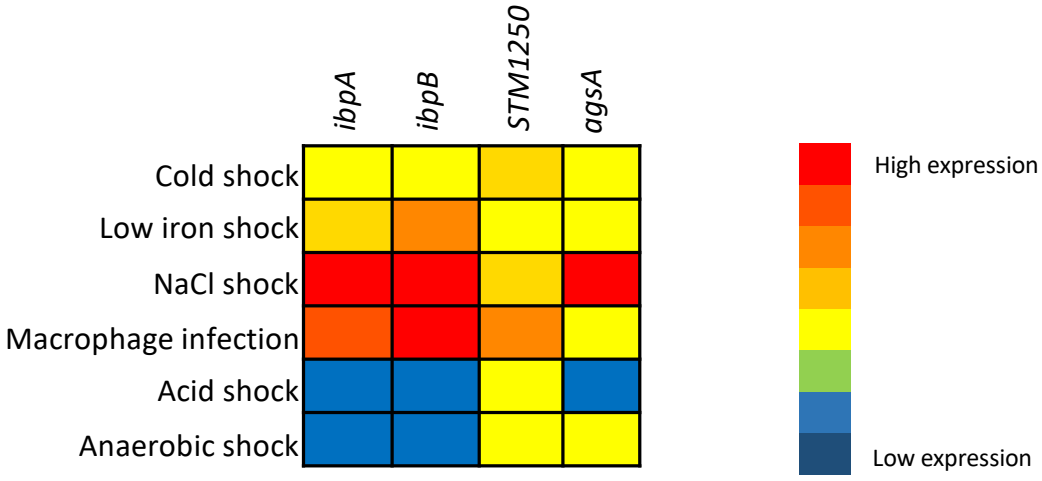


Figure 16. *ibpA*, *ibpB*, *agsA* and *STM1250* are differentially expressed during exposure of *Salmonella* to various environmental stresses. Environmental stress conditions were as follows: cold shock – 15 °C incubation, low iron shock – treatment with 0.2 mM 2,2' -dipyridyl treatment, salt shock – treatment with 0.3 mM NaCl, acid shock – exposure to pH 3 (HCl), anaerobic shock – incubation without agitation. Data extracted from the *Salmonella* gene expression compendium (Salcom) v1.0 (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?_HL) and v2.0 (http://bioinf.gen.tcd.ie/cgi-bin/salcom_v2.pl?_HL) (Kröger et al., 2013, Canals et al., 2019).

In addition to the Kröger et al. (2013) and Canals et al. (2019) studies, further genome-wide RNA sequencing (RNA-seq) and TraDIS studies have identified differential expression and mutant phenotypes associated with the *ibpA*, *ibpB*, *STM1250* and *agsA* genes. As summarised in Table 13, all four genes are upregulated under acid stress, the three sHsps (*ibpA, ibpB* and *agsA*) are upregulated

during macrophage intracellular infection and an *agsA* TraDIS mutant is attenuated in cattle.

It can be observed that differences in expression of the genes of interest exist between the data reported in Figure 16 and Table 13, for example all four genes are reported to be upregulated under acid stress in the Cao et al. (2019) study, but Kröger et al. (2013) reported low gene expression under acid stress. The differences in the parent *S.* Typhimurium strain, and the media and stress conditions utilised may have an impact on the phenotypes observed. These data emphasise the importance that stress response studies are conducted in a range of background strains, and a range of conditions.

Table 13. Phenotypes associated with IbpA, IbpB, STM1250 and AgsA in various *Salmonella* genome-wide screens.

Gene	*Salmonella* background strain[§]	Screen	Phenotype	Reference
ibpA	STM NBRC 12529	DNA microarray	Upregulated after 30-min recovery from heat injury	(Hsu-Ming et al., 2012)
	STM S6[#]	RNA-seq	Upregulated under acid stress	(Cao et al., 2019)
	STM SL1344	RNA-seq	Upregulated during macrophage infection	(Eriksson et al., 2003)
ibpB	STM NBRC 12529	DNA microarray	Upregulated after 60-min recovery from heat injury	(Hsu-Ming et al., 2012)
	STM S6	RNA-seq	Upregulated under acid stress	(Cao et al., 2019)
	STM SL1344	RNA-seq	Upregulated during macrophage infection	(Eriksson et al., 2003)
STM1250	STM NBRC 12529	DNA microarray	Upregulated after 30- and 60-min recovery from heat injury	(Hsu-Ming et al., 2012)
	STM S6	RNA-seq	Upregulated under acid stress	(Cao et al., 2019)
	Δ*fur*		Upregulated compared to WT	(Troxell et al., 2011)
	STM D23580 (iNTS)*	RNA-seq	Upregulated serum	(Ondari et al., 2019)
agsA	STM ST4/74	TraDIS	Mildly attenuated in cattle	(Chaudhuri et al., 2013)
	STM S6	RNA-seq	Upregulated under acid stress	(Cao et al., 2019)
	STM SL1344	RNA-seq	Upregulated during macrophage infection	(Eriksson et al., 2003)
	STM 14028	2DGE[$] proteomics	Protein downregulated in swarming cells compared to swimming cells	(Kim and Surette, 2004)

§ STM = *Salmonella* Typhimurium
# STM S6 is a novel isolate from chicken manure (Cao et al., 2019)
$ two-dimensional gel electrophoresis
* invasive non-typhoidal *Salmonella*

3.1.6 Aims

As outlined in the introduction to this chapter, IbpAB, AgsA and STM1250 are regulated by σ^E. Due to this shared regulation, we hypothesise that these proteins may contribute to cell envelope maintenance during exposure to different extracytoplasmic stresses. Therefore, this chapter investigates the contribution of the sHsps IbpA, IbpB and AgsA and the putative cytoplasmic protein STM1250 to the *Salmonella* envelope stress response. To date, the vast majority of work on sHsps and their functional partners has been performed on non-pathogenic *E. coli* strains such as *E. coli* K12. Moreover, our current understanding of the roles of such proteins in *Salmonella*, beyond that of survival against heat stress, is extremely limited. The lack of described phenotypes in the literature of *E. coli* and *Salmonella ibpAB* mutants indicates that in these deletion strains, other chaperones or sHsps may compensate for their loss. In *Salmonella* this is predicted to involve the sHsp AgsA and the putative cytoplasmic protein STM1250.

The aims of this chapter were to conduct a broad, preliminary phenotypic screen, exploring the roles of IbpAB, STM1250 and AgsA in *Salmonella* survival against a range of environmental stresses. Furthermore, we aimed to investigate our hypothesis of the existence of functional overlap between these proteins under stress-inducing conditions.

These aims were achieved as follows:

- Generation of single and combination gene deletion mutants, including a quadruple mutant (Δ*ibpAB*Δ*STM1250*Δ*agsA*) to assess potential functional redundancy.
- Analysis of bacterial growth at elevated temperatures and an investigation into the response of the mutant strains to heat and cold shock.
- A broad phenotypic screen, focussing on stresses that typically lead to damage to the OM and cell envelope, including sensitivity to vancomycin, polymyxin B, high salinity and metals.

3.2 Results

3.2.1 *E. coli* IbpA and IbpB are conserved differently among different bacterial species

The vast majority of current knowledge surrounding the IbpA/B sHsps has arisen from *E. coli* studies. In other bacterial species, where both proteins are present, they both share close amino acid sequence identity (97-99% for IbpA and 87-89% for IbpB) to *E. coli* IbpA and IbpB. Moreover, in bacteria that contain both genes, *ibpA* and *ibpB* form an operon and are separated by 110 bp (Gaubig et al., 2011). Interestingly, where only IbpA is present, as is the case for *Pseudomonas* and *Vibrio cholerae*, the sequence identity shared with *E. coli* IbpA is as low as 46% (Table 14).

Table 14. Percentage identity between *E. coli* IbpA/B and IbpA/B amino acid sequences from other bacteria. Amino acid sequence percent identity was determined by pairwise alignment (blastp, NCBI) and is shown for each pair. IbpA accession numbers are as follows: *E. coli* (NP_418142.1), *S.* Typhimurium (CBW19866.1), *V. cholerae* (AWB72831.1), *C. koseri* (QEU25751.1), *S. flexneri* (RIG77844.1) and *K. pneumoniae* (CDO11783.1). IbpB accession numbers are as follows: *E. coli* (NP_418142.1), *S.* Typhimurium (CBW19865.1), *C. koseri* (AYY76384.1), *S. flexneri* (KFZ98682.1) and *K. pneumoniae* (CDO11784.1).

Bacterial species pair	Percent identity (%) IbpA	Percent identity (%) IbpB
E. coli and *S.* Typhimurium	97	89
E. coli and *V. cholerae*	57	n/a
E. coli and *C. koseri*	99	89
E. coli and *P. aeruginosa*	46	n/a
E. coli and *S. flexneri*	99	100
E. coli and *K. pneumoniae*	97	87

3.2.2 Sequence similarities exist between the *Salmonella* IbpA, IbpB and AgsA sHsps

Pairwise amino acid sequence alignments were performed between IbpA, IbpB, STM1250 and AgsA using the EMBOSS Needle online tool (Madeira et al., 2019). Alignments are presented in Appendix D and sequence identity and homology for each pair are summarised in Table 15.

Table 15. *Salmonella* amino acid sequence identities and similarities following pairwise EMBOSS needle alignments between IbpA, IbpB, STM1250 and AgsA. Amino acid sequence percent identity and similarity was determined by pairwise alignment (EMBOSS needle) and is shown for each protein pair. Amino acid sequences were obtained from the NCBI database, accession numbers are as follows: IbpA (CBW19866.1), IbpB (CBW19865.1), STM1250 (CBW17281.1), AgsA (CBW17282.1).

Protein pair	Identity (%)	Similarity (%)
IbpA + IbpB	46.9	62.8
IbpA + STM1250	11.1	19.8
IbpA + AgsA	29.5	50.0
IbpB + STM1250	9.3	12.6
IbpB + AgsA	27.1	53.5
STM1250 + AgsA	9.1	17.2

As previously described, there is greater than 50% amino acid sequence homology between the sHsps. However, low homology was observed between the putative stress response protein STM1250 and the sHsps. Homology between the sHsps can be mostly attributed to the highly conserved α-crystallin domain – the N- and C-terminal domains are otherwise highly variable (Narberhaus, 2002). Therefore, since STM1250 lacks an α-crystallin domain this may explain why low homology is observed.

3.2.3 AgsA is highly conserved among *Salmonella* serovars while STM1250 is differentially conserved

The IbpA and IbpB sHsps are highly conserved across Gram-negative bacteria. Conversely, STM1250 and AgsA are specific to *Salmonella*. Analysis of the conservation of AgsA and STM1250 across *Salmonella* serovars revealed unique differences between the *Salmonella* specific proteins. Amino acid sequences were obtained from the NCBI database and multiple alignments were performed using M-Coffee (Notredame et al., 2000) and Expasy Boxshade (https://embnet.vital-it.ch/software/BOX_form.html). Seven additional serovars were selected for analysis, *S.* Typhimurium (SL1344), an invasive NTS serovar (D2580), two host-restricted enteric fever serovars *S.* Typhi (CT18) and *S*. Paratyphi (RKS4594) and four host generalists prevalent in the food chain, *S.* Choleraesuis (SC-B67), *S.* Enteritidis (SE95), *S.* Newport (SL254) and *S.* Dublin (CVM22429).

AgsA was identified in all serovars tested and shown to be a part of the core *Salmonella* genome (Figure 17).

Figure 17. AgsA amino acid alignment across selected *Salmonella* serovars shows that AgsA is highly conserved. Amino acid sequences were obtained from the NCBI database and alignments were performed using M-coffee online alignment tool. Accession numbers are as follows: *S* Typhimurium SL1344 (CBW17282.1), *S.* Typhimurium D23580 (CBG24270.1), *S.* Typhi CT18 (CAD02104.1), *S.* Paratyphi RKS4594 (ACN46598.1), *S.* Choleraesuis SC-B67 (AAX65168.1), *S.* Enteritidis SE95 (QIT85296.1), *S*. Newport SL254 (ACF65691.1), *S*. Dublin CVM22429 (AYB27868.1). Black indicates conserved residues and red indicates unconserved amino acids. Figure was generated using ExPasy Boxshade online tool.

Although also specific to *Salmonella,* STM1250 does not share significant similarity to other proteins in *Salmonella* nor in other Gram-negative bacteria. Interestingly, in contrast to AgsA, STM1250 was shown to be conserved differently across the *Salmonella* serovars tested in this study (Figure 18). There is between 99 and 100% amino acid sequence identity between *S.* Typhimurium serovars SL1344 and D23580 and *S.* Enteritidis. In addition, 92% sequence identity was shared between STM1250 from SL1344 and *S.* Typhi.

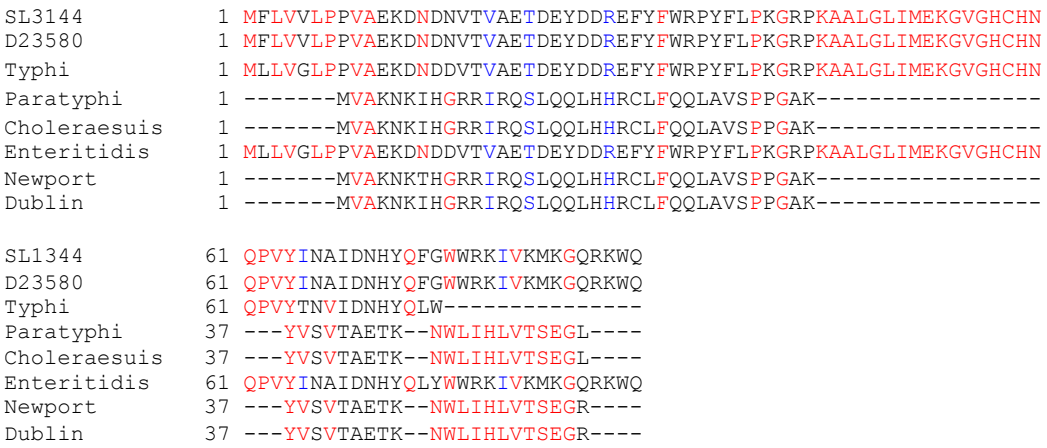


Figure 18. STM1250 amino acid alignment across selected *Salmonella* serovars shows that STM1250 is not equally conserved. Amino acid sequences were obtained from the NCBI database and alignments were performed using M-coffee online alignment tool. Accession numbers are as follows: *S* Typhimurium SL1344 (CBW17281.1), *S.* Typhimurium D23580 (CBG24269), *S.* Typhi CT18 (CAD02105.1), *S.* Paratyphi RKS4594 (ACN46599.1), *S.* Choleraesuis SC-B67 (AAX65167.1), *S.* Enteritidis SE95 (QIT82894), *S.* Newport SL254 (ACF64384), *S.* Dublin CVM22429 (AYB27869.1). Figure was generated using ExPasy Boxshade online tool. Figure from Hews et al. (2019b).

Table 16. Summary of STM1250 conservation between *S.* Typhimurium SL1344 and selected *Salmonella* serovars. Conservation of the STM1250 amino acid sequence between the SL1344 serovar and serovars of interest is depicted with a tick (✓) whilst a lack of conservation is depicted with a cross (×). Table is a summary of results presented in Figure 18.

***Salmonella* serovar**	**Conserved with STM1250 SL1344?**
S. Typhumurium D23580	✓
S. Typhi CT18	✓
S. Paratyphi RKS4594	×
S. Choleraesuis SC-B67	×
S. Enteritidis SE95	✓
S. Newport SL254	×
S. Dublin CVM22429	×

The STM1250 amino acid sequence of *S.* Typhimurium SL1344 was not highly conserved with *S.* Paratyphi, *S.* Choleraesuis, *S.* Newport or *S.* Dublin. Additionally, in *S.* Bongori, STM1250 was shown to be a pseudogene. Firstly, these data indicate that functional STM1250 arose following divergence of the *S. enterica* and *S. bongori* species and secondly, these data point towards a requirement and role for STM1250 in *Salmonella* mammalian infection. However, sequence conservation does not link STM1250 to a specific disease type such as invasive enteric fever nor does it point towards a role for STM1250 in a specific host.

The relationship between STM1250 protein sequences was further analysed through the construction of a phylogenetic tree, with MEGA 7 software (Hall, 2013). The divergence of the *S.* Typhimurium (SL1344 and D23580), *S.* Typhi and *S.* Enteritidis STM1250 protein sequence with that of *S.* Newport, *S.* Dublin, *S.* Paratyphi C and *S.* Choleraesuis is represented in the phylogenetic tree (Figure 19).

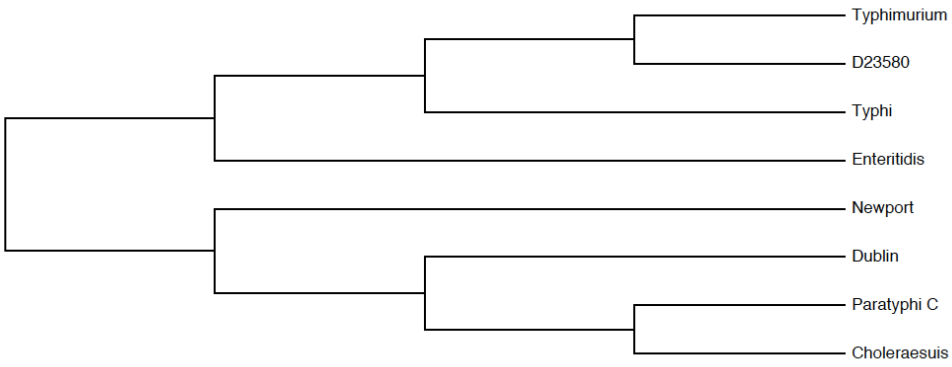


Figure 19. The diversity of STM1250 protein sequences may be associated with *Salmonella* serovar phylogenetic lineages. STM1250 amino acid sequences were obtained from the NCBI database and alignments were performed using Clustal W. The phylogenetic tree was constructed using MEGA 7, and the Maximum Likelihood method for estimating phylogenetic trees (Hall, 2013). Accession numbers are as follows: *S* Typhimurium SL1344 (CBW17281.1), *S*. Typhimurium D23580 (CBG24269), *S.* Typhi CT18 (CAD02105.1), *S.* Paratyphi RKS4594 (ACN46599.1), *S.* Choleraesuis SC-B67 (AAX65167.1), *S.* Enteritidis SE95 (QIT82894), *S*. Newport SL254 (ACF64384), *S*. Dublin CVM22429 (AYB27869.1).

3.2.4 Generation and verification of mutant strains

3.2.4.1 *Salmonella* SL1344 deletion mutants

Gene deletions were generated by lambda red recombination (Datsenko and Wanner, 2000) in the *S.* Typhimurium strain SL1344 using the mutagenesis primers in Table 2.7. Genes were replaced with a chloramphenicol or kanamycin resistance cassette, amplified from the pKD3 or pKD4 plasmids, respectively. External verification primers (Table 2.8) were used to verify that deletions had been correctly made and that the gene had been replaced with a chloramphenicol or kanamycin resistance cassette. Single gene deletions were moved into a clean (i.e. not carrying the pKD46 plasmid for lambda red recombination) WT SL1344 background by P22 transduction and mutation was confirmed by PCR. Verification of single mutants are presented in Figure 20 (Δ*ibpA*), Figure 21 (Δ*ibpB*), Figure 22 (Δ*STM11250*) and Figure 23 (Δ*agsA*)

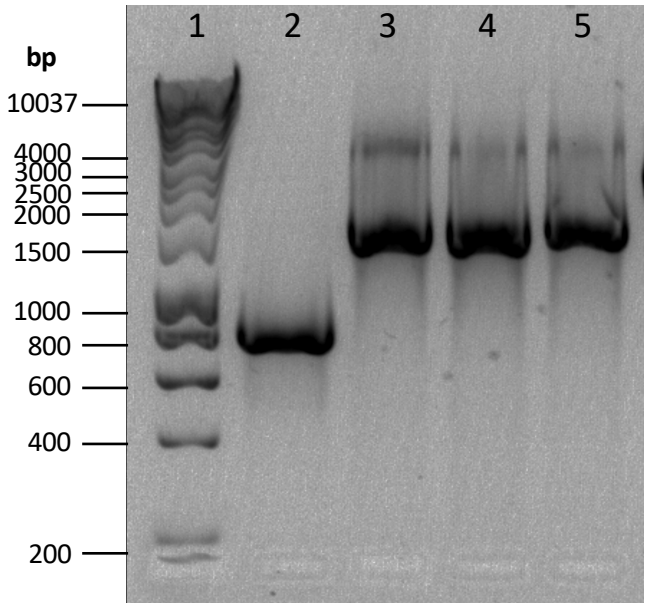


Figure 20. Verification of SL1344 *ibpA* mutant construction. PCR analysis was performed using *ibpA* external verification primers. Lane 1: DNA was run against DNA Hyperladder™ 1 kb (Bioline) for size comparison. Lane 2: WT *ibpA* gene, expected size 818 bp. Lanes 3-5: candidate *ibpA* chloramphenicol resistant mutants, size expected 1534 bp. DNA was separated on a 1% (w/v) agarose gel in TBE.

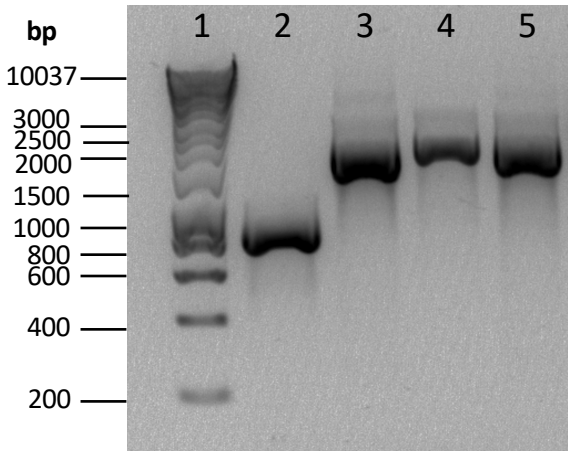


Figure 21. Verification of SL1344 *ibpB* mutant construction. PCR analysis was performed using *ibpB* external verification primers. Lane 1: DNA was run against DNA Hyperladder™ 1 kb (Bioline) for size comparison. Lane 2: WT *ibpB* gene, expected size 829 bp. Lanes 3-5: candidate *ibpB* chloramphenicol resistant mutants, size expected 1534 bp, Lanes 3 and 5 are the correct size. DNA was separated on a 1% (w/v) agarose gel in TBE.

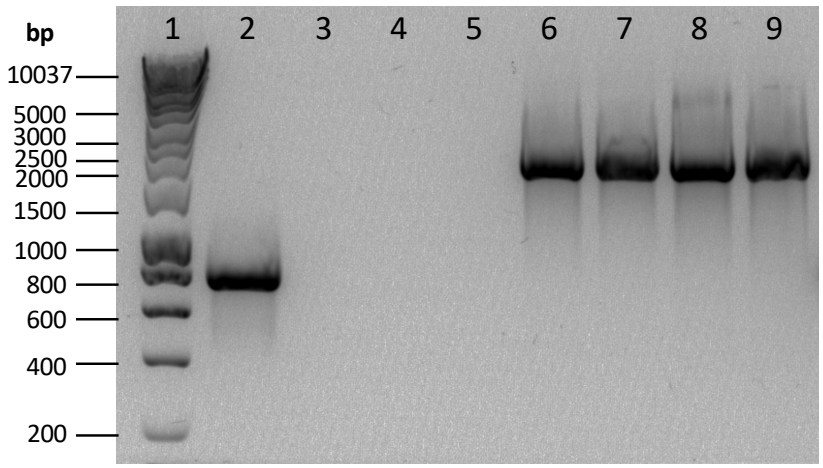


Figure 22. Verification of SL1344 *STM1250* mutant construction. PCR analysis was performed using *STM1250* external verification primers. Lane 1: DNA was run against DNA Hyperladder™ 1 kb (Bioline) for size comparison. Lane 2: WT *STM1250* gene, expected size 779 bp. Lanes 3-9: candidate *STM1250* kanamycin resistant mutants, size expected 1997 bp. DNA was separated on a 1% (w/v) agarose gel in TBE.

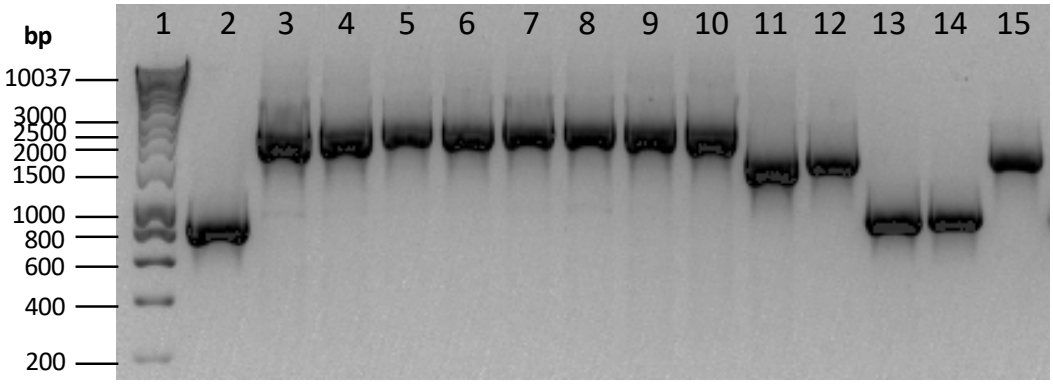


Figure 23. Verification of SL1344 *agsA* mutant construction. PCR analysis was performed using *agsA* external verification primers. Lane 1: DNA was run against DNA Hyperladder™ 1 kb (Bioline) for size comparison. Lane 2: WT *agsA* gene, expected size 870 bp. Lanes 3-10: candidate *agsA* kanamycin resistant mutants, size expected 1997 bp. Lanes 11-15: candidate *agsA* chloramphenicol resistant mutants, size expected 1534 bp (lanes 11, 12 and 15 are the correct size). DNA was separated on a 1% (w/v) agarose gel in TBE.

The close proximity of *ibpA* and *ibpB* allowed the Δ*ibpA*Δ*ibpB* (Δ*ibpAB*) double mutant to be generated in a single mutagenesis event. Again, the deletion was transferred to a clean WT background by P22 transduction and verified by PCR using the appropriate external primers (Table 2.8) (Figure 24).

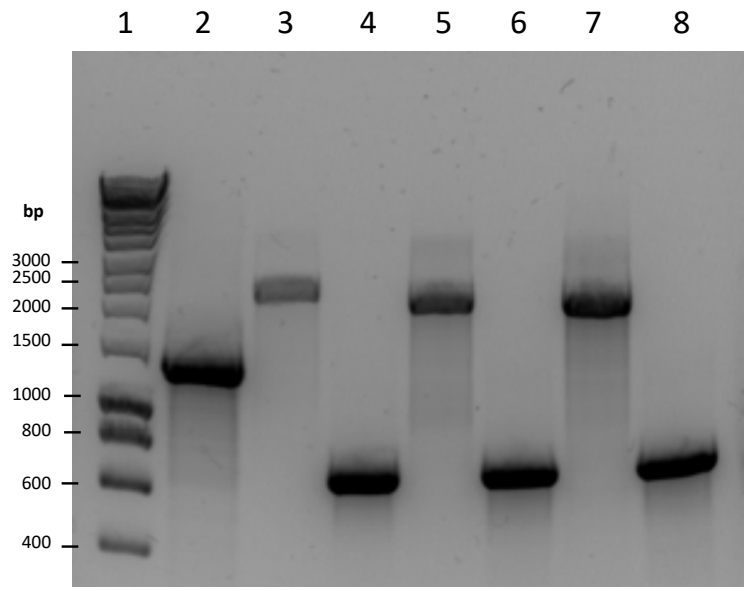


Figure 24. Verification of SL1344 *ibpAB* double mutant construction. PCR analysis was performed using *ibpAB* external verification primers and a kanamycin internal antibiotic resistance cassette primer. Lane 1: DNA was run against a DNA Hyperladder™ 1kB (Bioline) for size comparison. Lanes 2: WT *ibpAB* gene, expected size 1358 bp. Lanes 3, 5 and 7: *ibpAB* kanamycin resistant mutant, expected size 1997 bp. Lanes 4, 6 and 8: *ibpAB* mutant kanamycin cassette, expected size 699 bp. The isolated colonies in lanes 5 to 8 were the correct size. DNA was run on a 1% (w/v) agarose gel in TBE against Hyperladder™ 1 kb (Bioline).

The triple and quadruple deletion mutants were generated by P22 transduction following lambda red mutagenesis. For the triple mutant, Δ*ibpAB*Δ*agsA,* the *agsA* single deletion was transduced into the Δ*ibpAB* double mutant background. Two PCR reactions were performed for each candidate mutant to verify that the kanamycin gene was present at the *ibpAB* knockout site and the chloramphenicol resistance gene was present at the *agsA* deletion site (Figure 25).

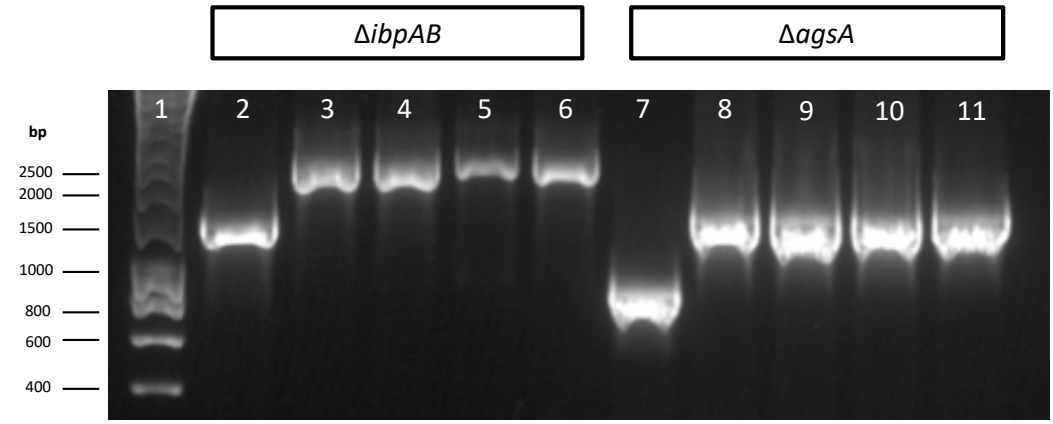


Figure 25. Verification of *ibpAB* and *agsA* deletions in the SL1344 triple (Δ*ibpAB*Δ*agsA*) mutant. PCR analysis was performed following P22 transduction of an Δ*agsA* mutation into a SL1344 Δ*ibpAB* mutant background. Lane 1: DNA was run on a 1% (w/v) agarose gel in TBE against Hyperladder™ 1 kb (Bioline). Lanes 2-6 correspond to PCR reactions with *ibpAB* external verification primers and lanes 7-11 correspond to PCR reactions with *agsA* external verification primers. Lane 2, WT *ibpAB*, expected size 1358 bp. Lane 3-6 candidate *ibpAB* kanamycin resistant mutants, expected size 1997 bp. Lane 7: WT *agsA*, expected size 870 bp. Lane 8-11 candidate *agsA* chloramphenicol resistant mutants, expected size 1534 bp.

For the quadruple deletion mutant, Δ*ibpAB*Δ*STM1250*Δ*agsA*, the Δ*STM1250*Δ*agsA* double mutant was transduced into the Δ*ibpAB* double knockout background. Two PCR reactions were performed in order to verify that mutant colonies contained the kanamycin gene at the *ibpAB* knockout site and the chloramphenicol gene at the *STM1250-agsA* knockout site. The resulting agarose gel electrophoresis analysis is presented in Figure 26.

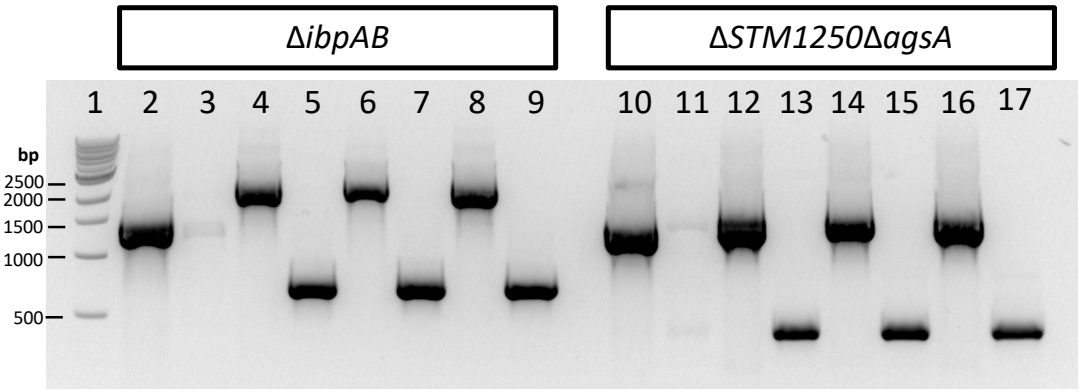


Figure 26. Verification of ibpAB, STM1250 and agsA deletions in the SL1344 quadruple (Δ*ibpAB*Δ*STM1250*Δ*agsA*) mutant. PCR analysis was performed following P22 transduction of Δ*STM1250*Δ*agsA* mutation into the Δ*ibpAB* mutant background. Multiple mutant colonies were selected Lane 1: DNA was run on a 1% (w/v) agarose gel in TBE against Hyperladder™ 1 kb (Bioline). Lanes 2-9 correspond to PCR reactions performed using *ibpAB* external verification primers or internal kanamycin antibiotic resistance cassette primers and lanes 10-17 correspond to PCR reactions performed using *STM1250-agsA* external verification primers or internal chloramphenicol antibiotic resistance cassette primers. Lane 2: WT *ibpAB*, expected size 1358 bp. Lane 3: WT kanamycin cassette, no band expected. Lane 4, 6, 8: *ibpAB* candidate mutants, expected size 1997 bp. Lane 5, 7, 9: *ibpAB* candidate mutants with kanamycin cassette primers, expected size 699 bp. Lane 10: WT *STM1250-agsA*, expected size 1292 bp. Lane 11: WT chloramphenicol cassette, no band expected. Lanes 12, 14, 16: Δ*STM1250-agsA* candidate mutants, expected size 1534 bp. Lane 13, 15, 17: Δ*STM1250-agsA* candidate mutants with chloramphenicol cassette primers, expected size 414 bp.

3.2.4.2 Generation of an *E. coli* K12 Δ*ibpAB* deletion mutant

For a comparison of the function of *ibpAB* in *Salmonella* with the closely related species *E. coli*, gene deletions were made in the *E. coli* MG1655 (K12) background strain by lambda red mutagenesis. As *for Salmonella*, the *ibpA* and *ibpB* genes were replaced with a kanamycin antibiotic resistance gene in a single mutagenesis event, amplified using primers in Table 2.7. Mutagenesis was confirmed using verification primers in Table 2.8 (Figure 27).

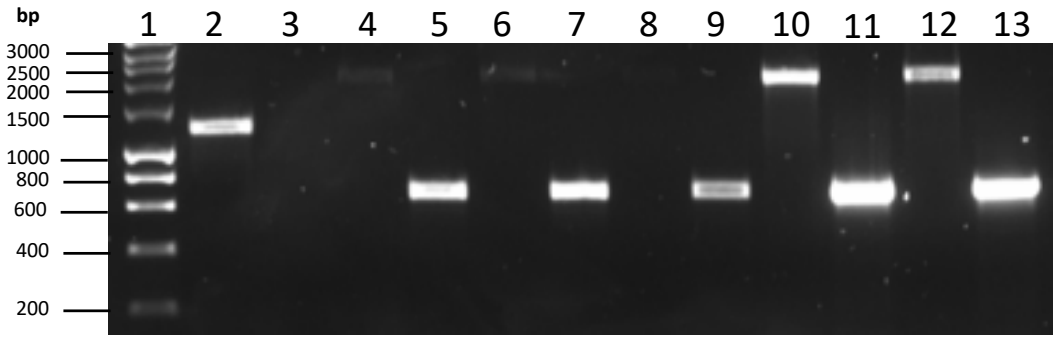


Figure 27. Verification of *ibpAB* deletion in the *E. coli* K12 background. PCR analysis was performed using *E. coli* specific *ibpAB* external verification primers. Lane 1: DNA was run on a 1% (w/v) agarose gel in TBE against Hyperladder™ 1 kb (Bioline). Lane 2: WT *ibpAB*, expected size 1259 bp. Lane 3: WT kanamycin cassette, no band expected. Lanes 4, 6, 8, 10, 12: candidate *ibpAB* mutants with external verification primers, size expected 1877 bp. Lanes 5, 7, 9, 11, 13: candidate *ibpAB* mutants with kanamycin cassette primers, size expected 619 bp. The isolated colony in Lane 10 was selected as a correct mutant.

3.2.5 The double and quadruple mutants grow equally to WT under aerobic and anaerobic conditions at 37 °C

To determine whether the deletion of *ibpA*, *ibpB*, *STM1250* and *agsA* resulted in any overall growth defects in *S.* Typhimurium, the double and quadruple mutant strains were grown either aerobically at 37 °C in rich LB medium or anaerobically at 37 °C in minimal (M9) medium, sparged with N_2 gas, using glycerol as a carbon source and sodium nitrate as the terminal electron acceptor. For aerobic growth, cultures were incubated with aeration and OD_{600} readings were taken hourly for 8 hours. For anaerobic growth, cultures were incubated statically for 48 hours and OD_{600} readings were taken at 17, 24, 40 and 48 hours.

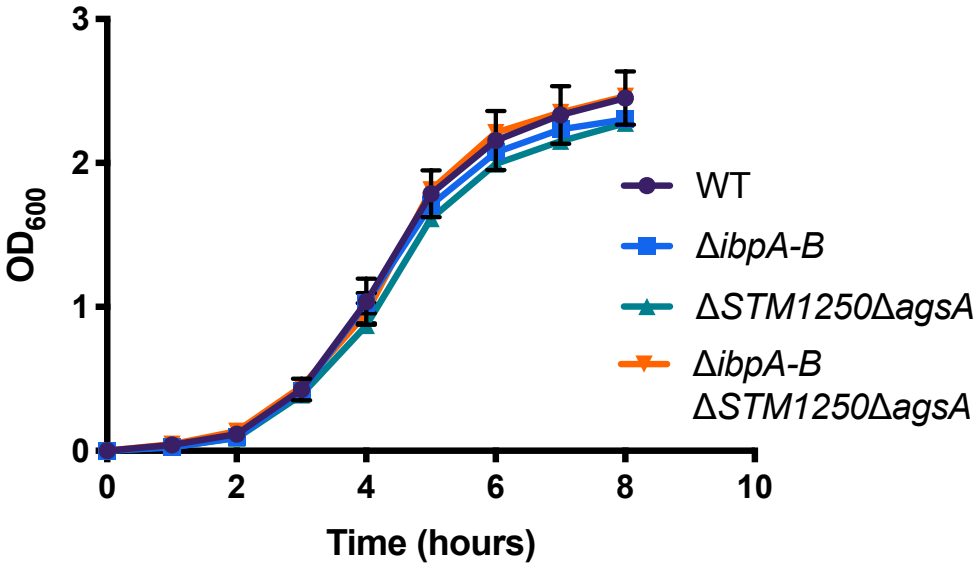


Figure 28. WT and mutant strains grow equally at 37 °C in LB. Bacteria were grown in 50 mL LB with aeration at 220 rpm. OD_{600} readings were taken hourly. Data points are the means of three separate experiments performed in duplicate and error bars represent SEM.

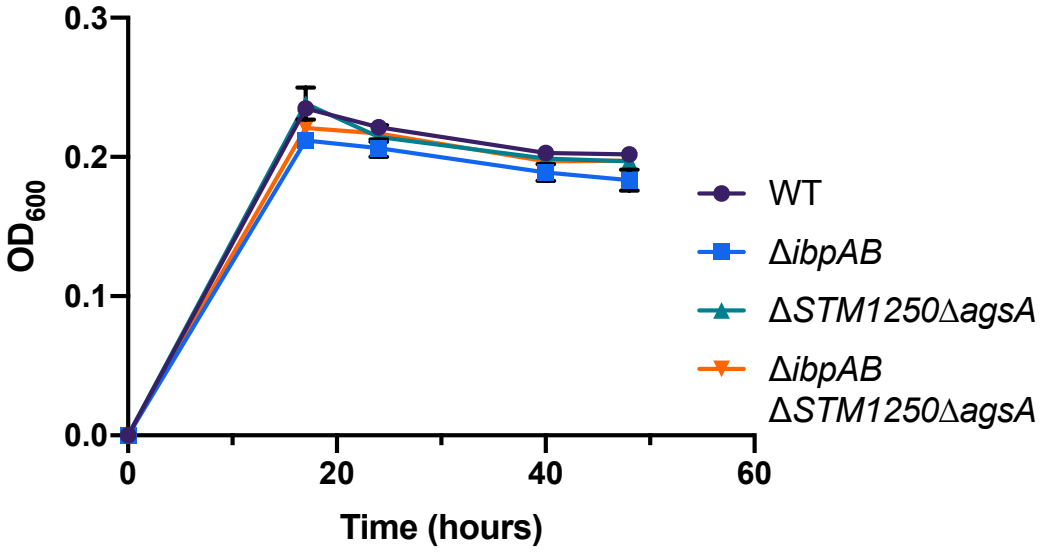


Figure 29. *ibpAB*, *STM1250* and *agsA* are not required for anaerobic growth of *S.* Typhimurium. Bacteria were grown anaerobically at 37 °C in 200 mL M9 minimal media containing 20 mM sodium nitrate ($NaNO_3$). Samples were taken at 17, 24, 40 and 48 hours and the OD_{600} measured. Data points are the means of three separate experiments performed in duplicate and error bars represent SEM.

Under both aerobic (Figure 28) and anaerobic conditions (Figure 29), there was no difference in the overall growth of the *Salmonella* mutants in comparison to the WT. Since the aims of this study were to assess the ability of the mutant strains to survive against environmental stresses, it could be confirmed that phenotypic differences in future experiments were not attributed to overall growth defects of the mutants.

3.2.6 IbpAB and AgsA, but not STM1250, are involved in *Salmonella* resistance to heat

Previous studies have shown that the double deletion of *ibpAB* in *E. coli* does not affect survival following heat shock at extreme temperatures of 50 °C (Thomas and Baneyx, 1998). Despite this, IbpA and IbpB have been shown to interact with protein aggregates following heat shock and are therefore understood to play a role in the bacterial heat shock response (Laskowska et al., 1996). We sought to verify the importance of *ibpA* and *ibpB* in *Salmonella* through investigation of the survival of Δ*ibpA* and Δ*ibpB* single mutants and an Δ*ibpAB* double mutant at 50 °C. Additionally, we aimed to determine the contribution of the *Salmonella* specific sHsp AgsA and the putative cytoplasmic protein STM1250 to the heat shock response. Studies focussing on the roles of these proteins in *Salmonella* are limited, although it has been shown that a triple Δ*ibpAB*Δ*agsA* mutant is significantly more sensitive to heat shock at 70 °C than WT, Δ*ibpAB* or Δ*agsA* mutants (Tomoyasu et al., 2003).

Bacteria were cultured at 37 °C to mid-exponential phase, the cultures were then transferred to 50 °C for up to 4 hours. Following a temperature upshift from 37 °C to 50 °C, the survivals of the single deletion mutants (Δ*ibpA*, Δ*ibpB*, Δ*STM1250* and Δ*agsA*) were unaffected at both 2 hours and 4 hours post-heat shock compared to WT, with the survival rate of all strains being in the range of 70% – 80% after 4 hours (Figure 30).

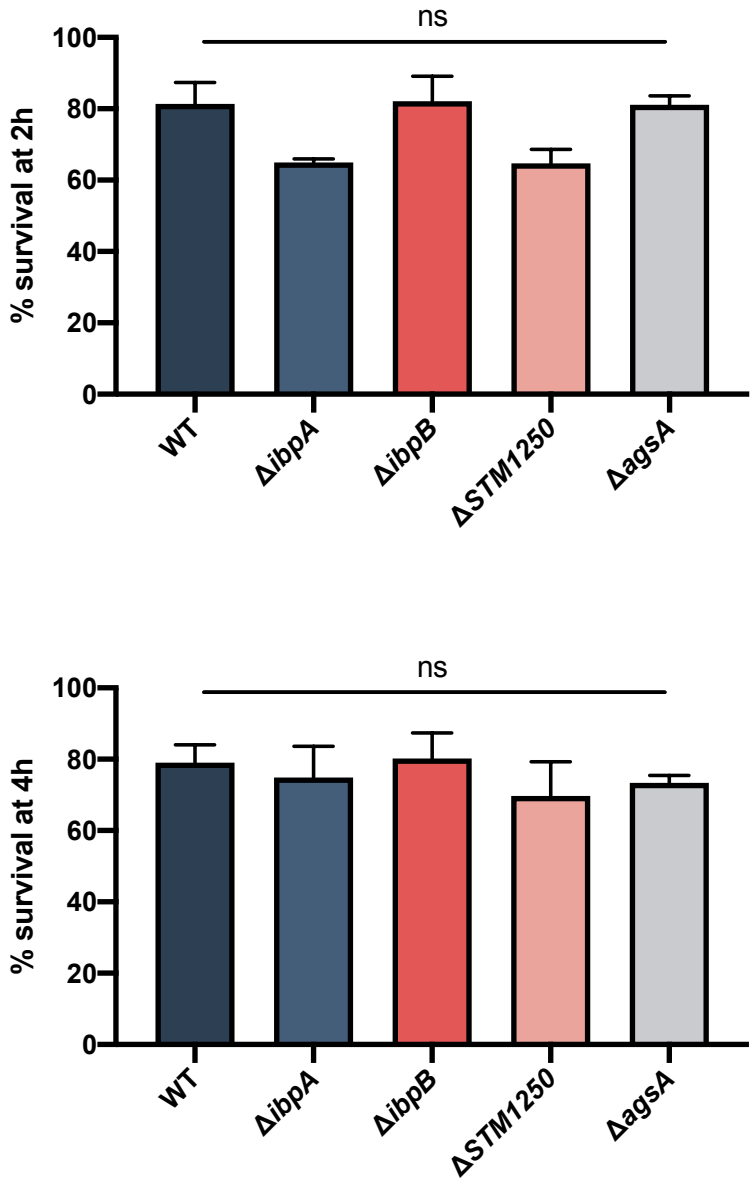


Figure 30. *ibpA, ibpB, STM1250* and *agsA* single gene deletion mutants are not susceptible to heat shock at 50 °C after 2 hours or 4 hours. Bacteria were grown in LB at 37 °C for 3 hours before incubation at 50 °C for 2 hours or 4 hours. Data are the means of three separate experiments performed in duplicate. Error bars represent SEM. Data was analysed by one-way ANOVA with Tukey's multiple comparisons test, ns $p > 0.05$

In a similar manner to the response of the single mutants, and in agreement to previous *ibpAB* studies in *E. coli*, the survival of the *Salmonella* Δ*ibpAB* double mutant was unchanged compared to WT after 2 hours and 4 hours incubation at 50 °C (Figure 31A and B). Similarly, survival of the Δ*STM1250*Δ*agsA* double mutant was

unchanged compared to the WT at both time points sampled (Figure 31A and B). The additive effect of deleting *agsA* and *STM1250-agsA* in the triple and quadruple deletion mutants, respectively, did not affect percentage survival compared to WT after 2 hours (Figure 31C). However, the triple deletion mutant displayed a significant two-fold reduction, compared to WT, in percentage survival after the 4 hour incubation at 50 °C (Figure 31D). The percentage survival of the quadruple mutant survival was also reduced two-fold compared to WT after 4 hours (Figure 31D); however, there was no difference observed between the triple and quadruple mutant, suggesting that deletion of STM1250 does not cause a further additive effect.

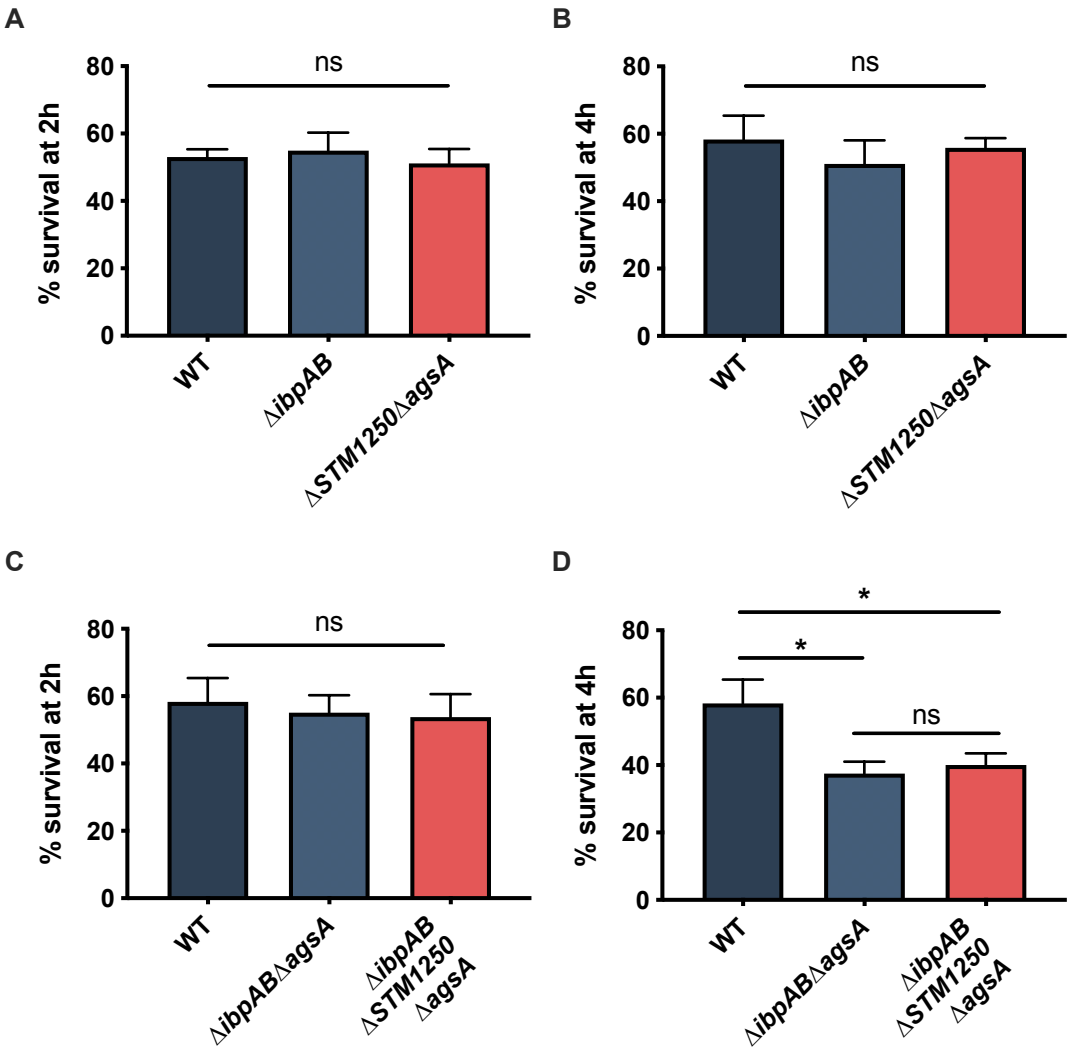


Figure 31. Deletion of *ibpA*, *ibpB* and *agsA*, but not *STM1250*, significantly reduces survival of *Salmonella* after heat shock at 50 °C for 4 hours. Double deletion mutants Δ*ibpAB* and Δ*STM1250*Δ*agsA* are unaffected by 50 °C heat shock after 2 hours (A) or 4 hours (B). The survival rates of the triple (Δ*ibpAB*Δ*agsA*) and quadruple (Δ*ibpAB*Δ*STM1250*Δ*agsA*) deletion mutants are unaffected by 50 °C heat shock after 2 hours (C) but are significantly reduced compared to WT following sustained heat stress at 50 °C for 4 hours (D). Bacteria were grown in LB at 37 °C for 3 hours before incubation at 50 °C for 2 hours or 4 hours. Data are the means of three separate experiments performed in duplicate. Error bars represent SEM. Data was analysed by one-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$.

In addition to the heat shock assays, bacteria were also assessed for their ability to grow at a sustained high temperature (exposed to high temperature from the lag phase as opposed to mid-exponential phase in the heat shock assays). Bacteria were

grown in LB media at 47 °C for 8 hours and OD_{600} measurements were taken hourly. During incubation at 47 °C, overall growth of all strains was slower and reached a final OD_{600} lower than that at 37 °C (Figure 28) (a final OD_{600} of 1.5 was observed at 47 °C compared to a final OD_{600} of 2.5 at 37 °C). Despite this, there was no significant difference between the growth of the WT or mutant strains (Figure 32).

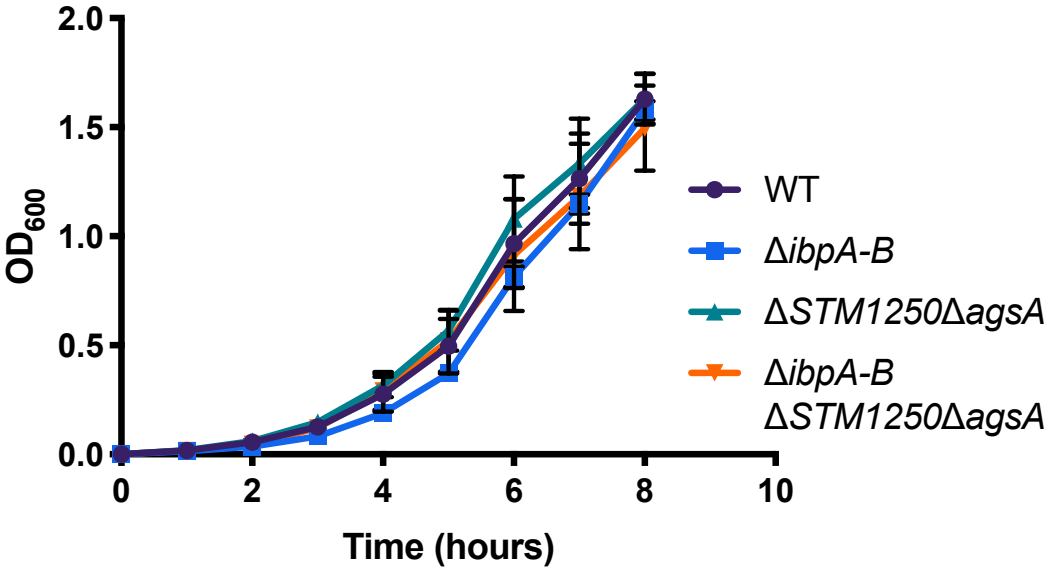


Figure 32. WT and deletion mutants grow equally at 47 °C. Bacteria were grown in 50 mL LB with aeration at 47 °C for up to 8 hours. Samples were taken hourly and OD_{600} readings taken. Data points are the means of three separate experiments performed in duplicate. Error bars represent SEM.

3.2.7 The small heat shock proteins IbpA, IbpB and AgsA are important for resistance to cold shock

Bacterial adaptation to cold shock and prolonged cold stress is of particular significance to foodborne pathogens such as *Salmonella*. The ability to survive at low temperatures, such as those used in refrigeration of food products, advances the passage of *Salmonella* through the food chain. Cold shock is a key inducer of the *rpoE* regulon (Miticka et al., 2003) and bacterial membrane fluidity is disrupted following incubation at low temperatures (Phadtare, 2004, Phadtare and Severinov, 2010, Fan and Evans, 2015, Keto-Timonen et al., 2016). Considering the heat shock sensitivity of the Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants, the regulation

of the genes of interest by σ^E and their hypothesised roles in maintenance of membrane integrity, survival against cold shock was also investigated.

To determine the role of the sHsps and STM1250 in the survival of *Salmonella* following cold shock, bacteria were grown to mid-exponential phase before being transferred to 10 °C for up to 2 hours. The 2-hour incubation was shown to be bacteriostatic for all strains tested and growth was limited (Figure 33). Interestingly, after the first 30 minutes of incubation at 10 °C, growth of the WT was significantly increased, by approximately two-fold, compared to the Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants, indicating a possible additional role for the sHsps in the growth and survival during the initial exposure to low temperatures (Figure 33). In contrast, overall, and at each time point sampled, there was no difference between the survival of the Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants. This indicated, in a similar manner to the heat shock results, that STM1250 does not function as a temperature-responsive protein in *Salmonella*.

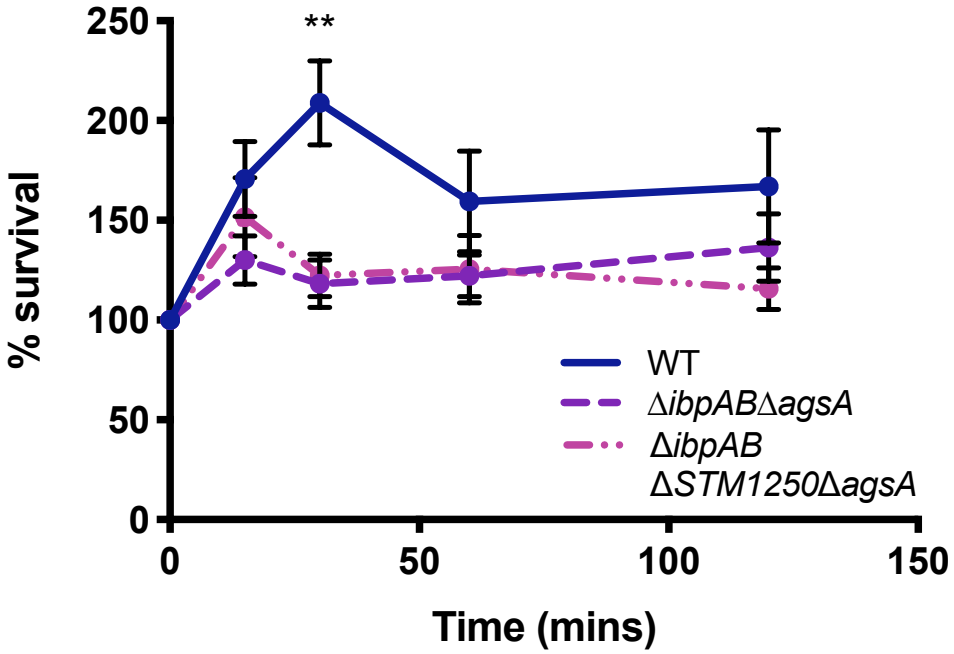


Figure 33. *ibpAB* and *agsA*, but not *STM1250*, contribute to *Salmonella* survival after 30 mins cold shock at 10 °C. Bacteria were grown to mid-exponential phase at 37 °C and then transferred to 10 °C for up to 2 hours. Samples were taken at 0, 15, 30, 60 and 120 mins, serially diluted and plated for determination of surviving CFU/mL. Data points represent the mean of three separate experiments performed in duplicate and error bars are SEM. Data analysed by one-way ANOVA with Tukey's post-test. ** $p < 0.005$.

3.2.8 IbpAB, STM1250 and AgsA are not required for metal resistance in *Salmonella*

Multiple studies have identified potential involvement of bacterial sHsps in metal resistance (Pérez et al., 2007, Matuszewska et al., 2008, Ezemaduka et al., 2018, Lv et al., 2019). To this end, in this study we sought to determine whether IbpA, IbpB, STM1250 or AgsA are involved in the resistance to metals, specifically copper and zinc.

3.2.8.1 Copper

Copper is an important micronutrient; however, in excess, copper ions (Cu^{+} and Cu^{2+}) are highly toxic to bacteria (Ladomersky and Petris, 2015). Bacteria possess important copper detoxification systems, required to maintain appropriate homeostasis of intracellular ions (Bondarczuk and Piotrowska-Seget, 2013, Cao et al., 2019, Giachino and Waldron, 2020). The antibacterial properties of copper are

routinely used in healthcare settings and the food industry, specifically in the rearing of poultry, as a growth promoter and to prevent enteric infection (Arias and Koutsos, 2006, Gardner and Olson, 2018). Understanding bacterial copper resistance could provide valuable insights into the development of alternative therapeutics.

Previously, deletion of *ibpA* and *ibpB* has been shown to reduce the overall growth and survival of *E. coli* in the presence of 3 mM copper chloride ($CuCl_2$) (Matuszewska et al., 2008). In order to investigate the contribution of IbpA and IbpB, in addition to STM1250 and AgsA, to the copper resistance of *Salmonella*, bacteria were grown in LB media at 37 °C containing either 2 mM (Figure 34), 3 mM (Figure 35) or 4 mM (Figure 36) $CuCl_2$ in a plate reader for 24 hours and OD_{600} readings were taken hourly.

The overall growth of all strains was reduced by the addition of $CuCl_2$ in a concentration dependent manner. However, there was no significant difference in the overall growth of the WT or the mutants at all concentrations tested.

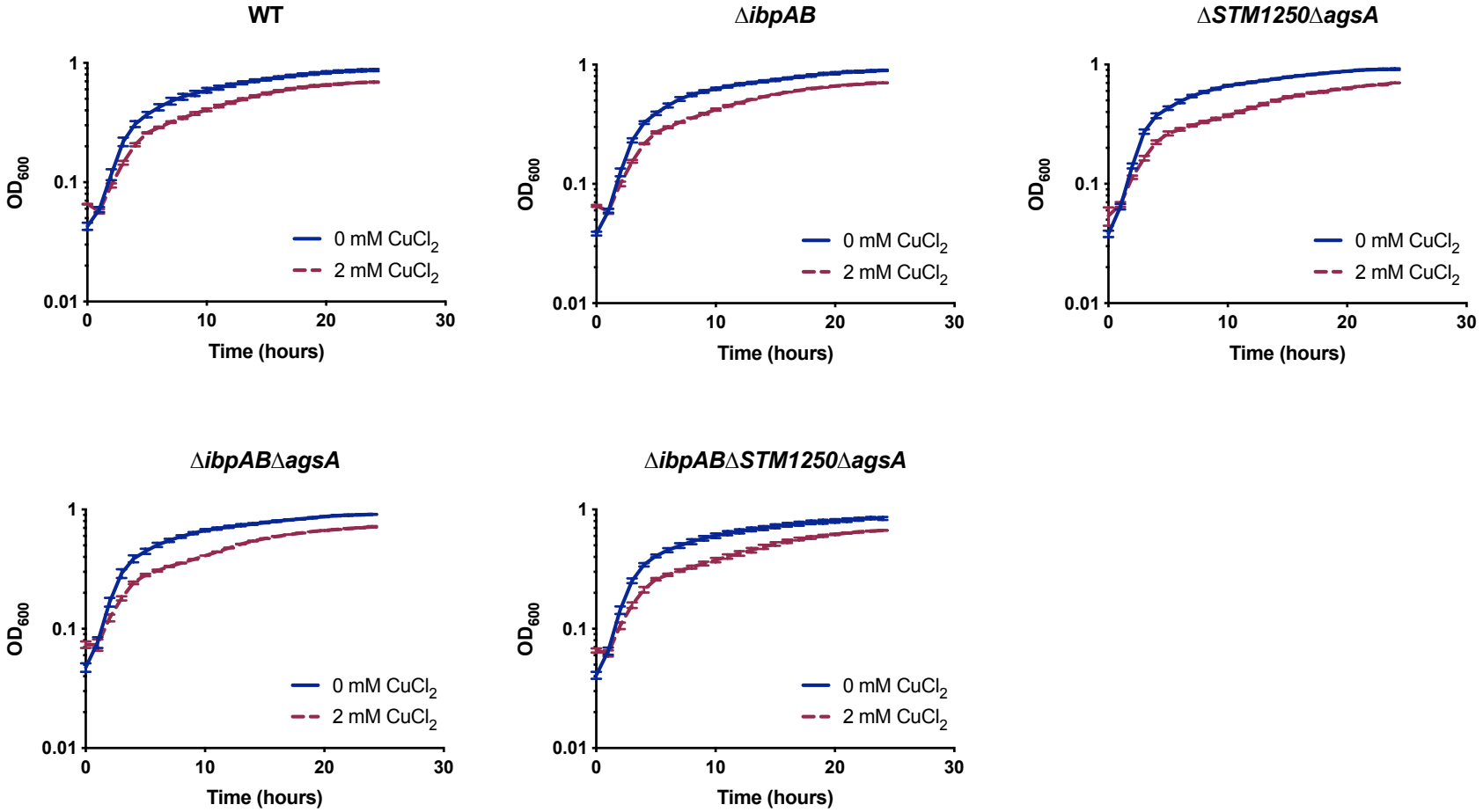


Figure 34. *ibpAB*, *STM1250* and *agsA* are not required for growth in 2 mM copper chloride ($CuCl_2$). Bacteria were grown in 24-well plates, aerobically in LB containing 2 mM $CuCl_2$ at 37 °C for 24 hours. OD_{600} readings were taken hourly by a SpectroMax M5 plate reader (Molecular Devices). Data points representative of the means of three separate experiments performed in duplicate. Error bars represent SEM and where not visible are smaller than graph symbols.

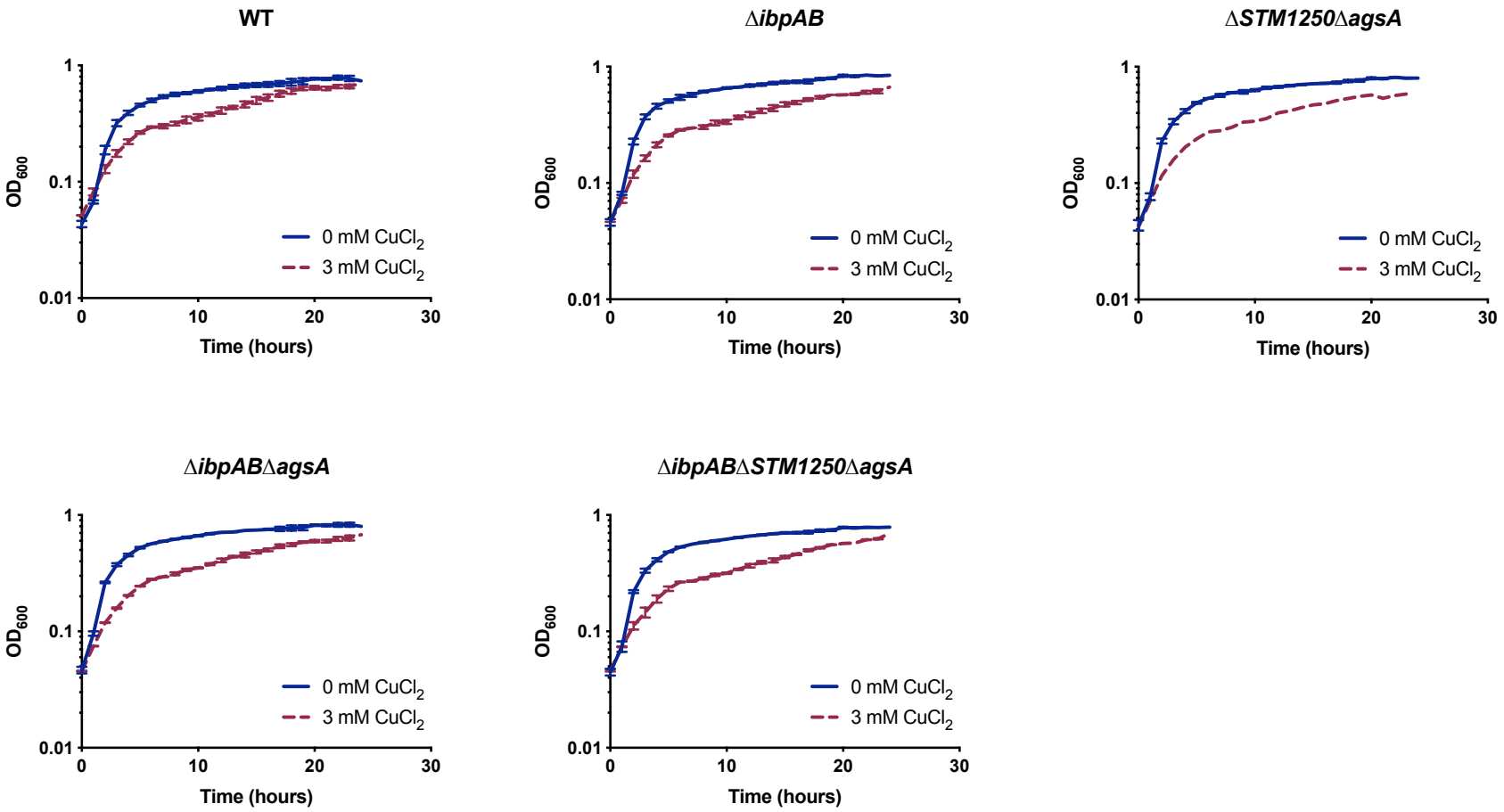


Figure 35. *ibpAB*, *STM1250* and *agsA* are not required for growth in 3 mM copper chloride ($CuCl_2$). Bacteria were grown in 24-well plates, aerobically in LB containing 3 mM $CuCl_2$ at 37 °C for 24 hours. OD_{600} readings were taken hourly by a SpectroMax M5 plate reader (Molecular Devices). Data points representative of the means of three separate experiments performed in duplicate. Error bars represent SEM and where not visible are smaller than graph symbols.

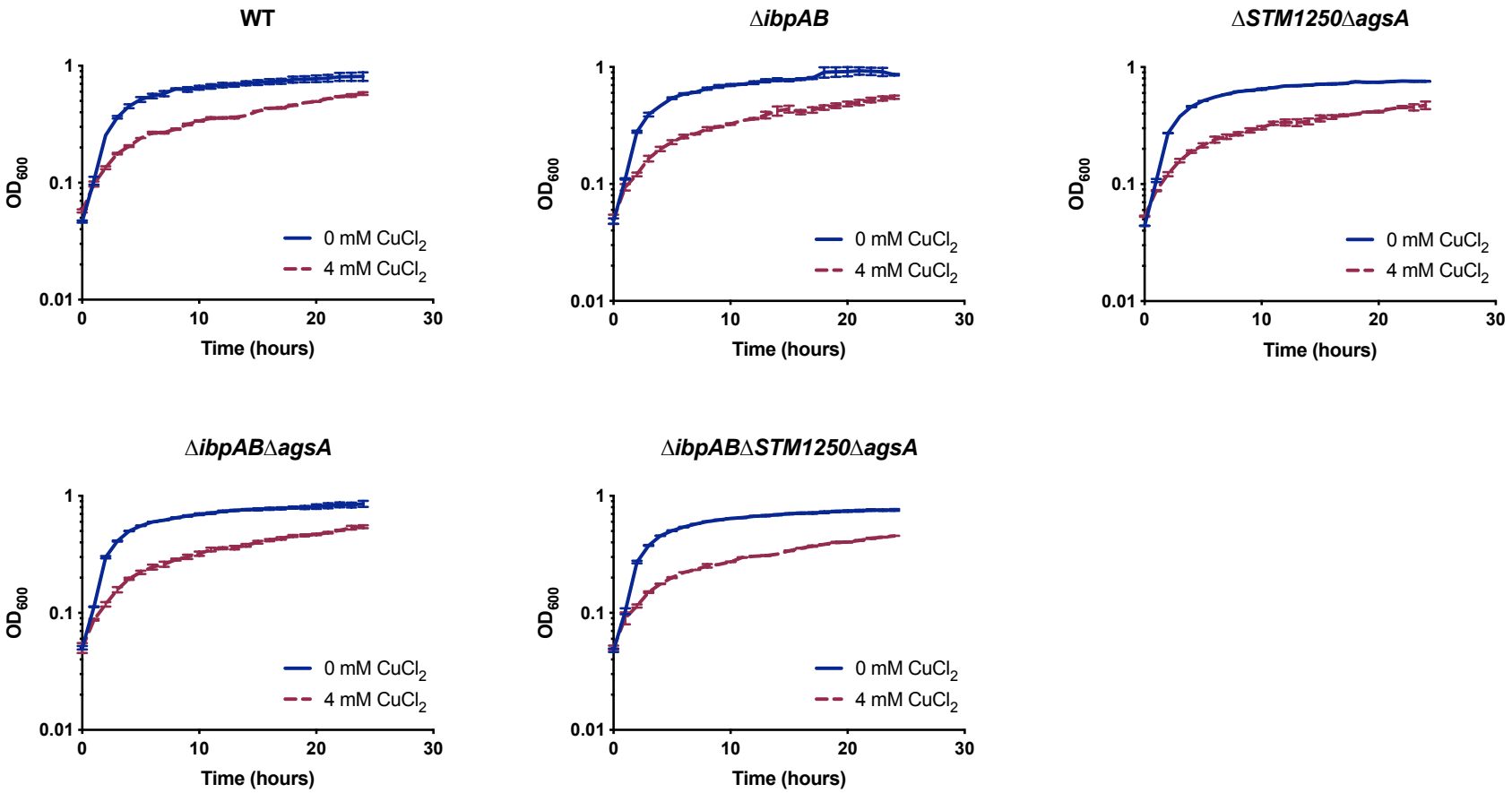


Figure 36. *ibpAB*, *STM1250* and *agsA* are not required for growth in 4 mM copper chloride ($CuCl_2$). Bacteria were grown in 24-well plates, aerobically in LB containing 4 mM $CuCl_2$ at 37 °C for 24 hours. OD_{600} readings were taken hourly by a SpectroMax M5 plate reader (Molecular Devices). Data points representative of the means of three separate experiments performed in duplicate. Error bars represent SEM and where not visible are smaller than graph symbols.

In order to statistically confirm whether $CuCl_2$ affected the ability of WT and mutant *Salmonella* to grow, the initial growth rates of the WT and mutant strains at each copper concentration were calculated (Figure 37). The growth rates confirmed that deletion of the genes of interest in this study do not affect *Salmonella* copper sensitivity during aerobic growth (Figure 37).

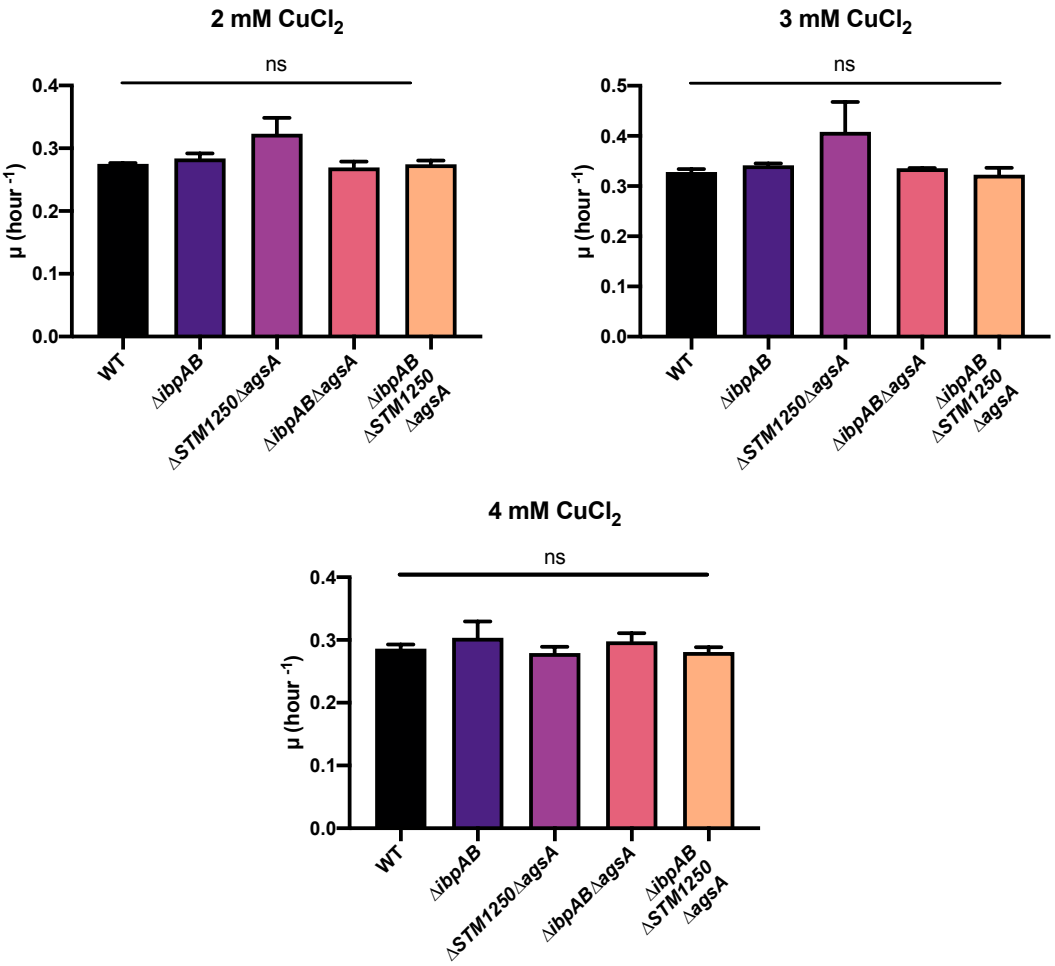


Figure 37. The initial growth rate (μ hour^{-1}) of mutant *S.* Typhimurium is unaffected by 2 mM, 3 mM or 4 mM copper chloride ($CuCl_2$) in comparison to the WT. The initial growth rate was calculated for the first 2 hours of growth in the presence of different concentrations of $CuCl_2$, as indicated above each graph. Data are the means of three separate experiments performed in duplicate and error bars represent SEM. Data was analysed by one-way ANOVA with Tukey's post-test. ns $p > 0.05$.

Sensitivity to copper chloride was also tested by growth of bacteria on LB agar containing 4 mM copper chloride. For this purpose, as an initial screen only the WT and quadruple mutant were tested. Colonies were counted after 48 hours incubation at 37 °C and CFU/mL were compared to colonies grown on LB agar only (no copper chloride control).

Overall, in agreement with the growth curve data, there was no significant difference between the percentage survival on $CuCl_2$ agar between the WT and the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant (Figure 38). Since no difference was observed between these two strains, the remaining deletion mutants were not tested.

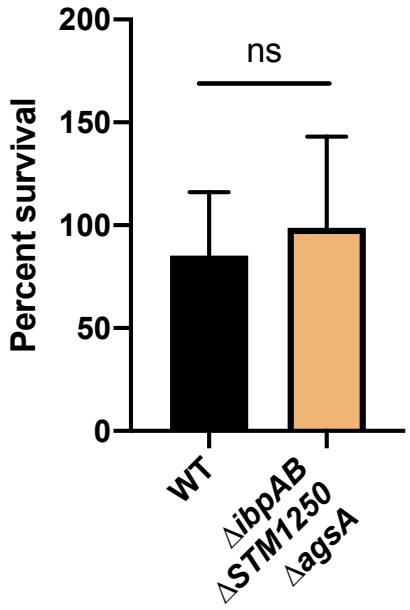


Figure 38. The Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is not sensitive to 4 mM copper chloride in LB agar. Bacteria were grown on LB agar containing 4 mM copper chloride and surviving CFU/mL were compared to growth on LB only. Data are the means of three separate experiments performed in duplicate. Data was analysed by Student's *t*-test, ns $p > 0.05$.

The differences between the data in this study and previously published data with a role for IbpAB in *E. coli* resistance to copper were intriguing and warranted further investigation. An Δ*ibpAB* mutant in the *E. coli* K12 background strain was generated (3.2.4.2) and its sensitivity to copper was investigated. For this, the method employed by Matuszewska et al. (2008) was used and the WT and Δ*ibpAB* mutant *Salmonella*

(SL1344) and *E. coli* (K12) strains were subjected to treatment with either 3 mM or 4 mM copper.

As described by Matuszewska et al. (2008), bacteria were grown to mid-exponential phase at 30 °C before the addition of 3 mM or 4 mM copper sulphate ($CuSO_4$). Cells were then incubated for a further 18 hours at 30 °C and the overall growth of each sample determined by measurement of the OD_{600}. Treatment with 3 mM $CuSO_4$ resulted in a slight reduction of the K12 Δ*ibpAB* mutant compared to WT but this was not deemed to be a significant difference following statistical (*t*-test) analysis. Equally, there was no difference between the *S.* Typhimurium WT and Δ*ibpAB* mutant strain. Following treatment with 4 mM $CuSO_4$, overall growth of the WT and mutant K12 strains were two-fold lower compared to treatment with 3 mM $CuSO_4$. Interestingly, *Salmonella* was not as severely affected and again, there was no difference between growth of the WT and mutant strain.

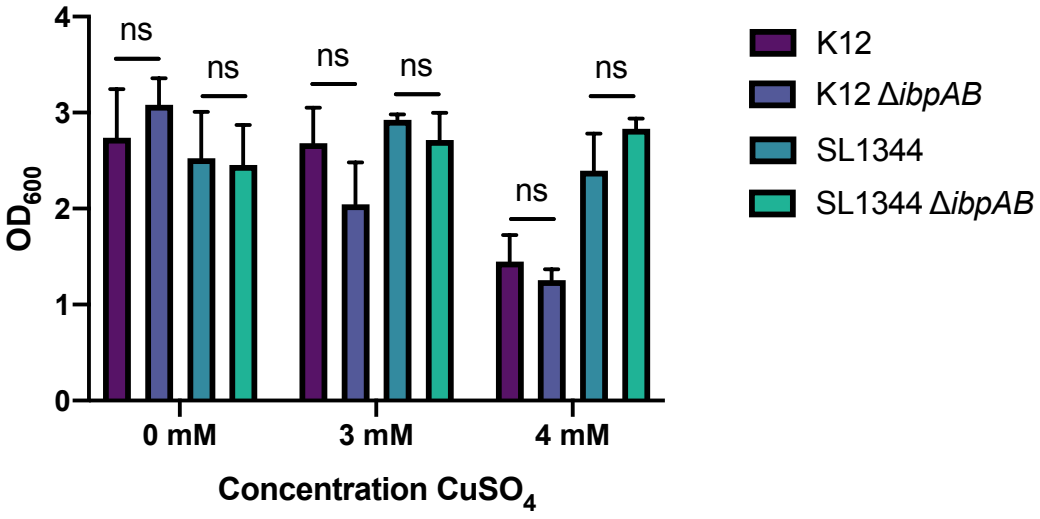


Figure 39. Growth of *E. coli* K12 Δ*ibpAB* but not SL1344 Δ*ibpAB* is impaired in the presence of $CuSO_4$. Bacteria were grown at 30 °C to mid-exponential phase before the addition of $CuSO_4$. Cells were incubated O/N at 30 °C for a further 18 hours and the resulting OD_{600} was measured. Data are the means of three separate experiments and error bars are SEM. Statistical analysis was performed by Student's *t*-test, ns $p > 0.05$.

Previously, heterologous overexpression of AgsA in *E. coli* has been shown to increase *E. coli* resistance to copper (Lv et al., 2019). In this study, *STM1250* and *agsA* were cloned into the IPTG-inducible overexpression vector pET-Duet1 to

produce the vectors p*STM1250* and p*STM1250-agsA*. The constructs were transformed into *E. coli* K12 WT and Δ*ibpAB* and the effect of STM1250/AgsA overexpression on the resistance to copper was investigated. Bacteria were grown to mid-exponential phase and treated with 3 mM or 4 mM $CuSO_4$ as previously. At the point of adding $CuSO_4$, cultures were also supplemented with 1 mM IPTG to overexpress the STM1250 and AgsA proteins from the pET-Duet vector. The results presented in Figure 40 show that overexpression of STM1250 (p*STM1250*) or STM1250 and AgsA (p*STM1250-agsA*) was unable to increase survival of *E. coli* in the presence of 3 mM $CuSO_4$. In fact, in the presence of 4 mM $CuSO_4$, overall growth of *E. coli* expressing STM1250 and STM1250-AgsA was significantly reduced in comparison to WT. Since *E. coli* is already under stress in the presence of 4 mM $CuSO_4$, the additive effect of the demand of heterologous protein overproduction may contribute to the growth defects observed.

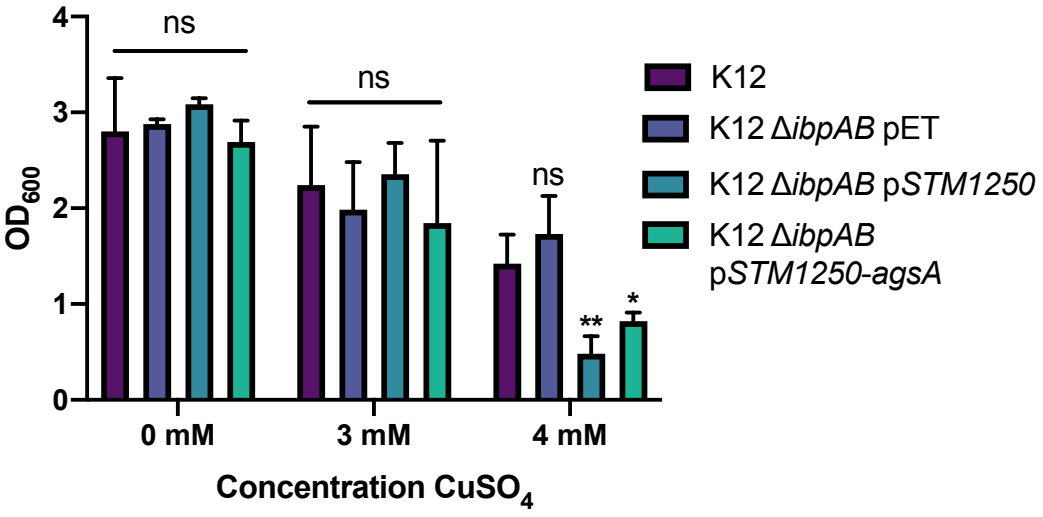


Figure 40. Overexpression of AgsA and/or STM1250 does not increase growth of *E. coli* Δ*ibpAB* in the presence of copper sulphate ($CuSO_4$). Bacteria were grown at 30 °C to mid-exponential phase before the addition of $CuSO_4$ and 1 mM IPTG. Cells were incubated O/N at 30 °C for a further 22 hours and the resulting OD_{600} was measured. Data are the means of two separate experiments. Statistical analysis was performed by one-way ANOVA with Tukey's post-test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$. Error bars are SEM.

3.2.8.2 Zinc chloride

In addition to copper, previous work has identified that the heterologous expression of AgsA in *E. coli* can provide resistance to zinc. Ezemaduka et al. (2018) demonstrated that the half maximal effective concentration (EC_{50}) of zinc increased 5-fold in *E. coli* overexpressing AgsA in comparison to WT. In addition, previous studies have shown that *ibpA* and *ibpB* are upregulated by the ZraSR zinc responsive TCS (Wells, 2015). Zinc is essential for *Salmonella;* however, like copper in excess zinc is toxic and can compete with other metals for cofactor binding sites, catalyse disulphide bond formation and cause generation of reactive oxygen species (Djoko et al., 2015).

As an initial screen for zinc sensitivity, the susceptibility of the quadruple mutant to increasing concentrations of zinc chloride ($ZnCl_2$) was investigated.

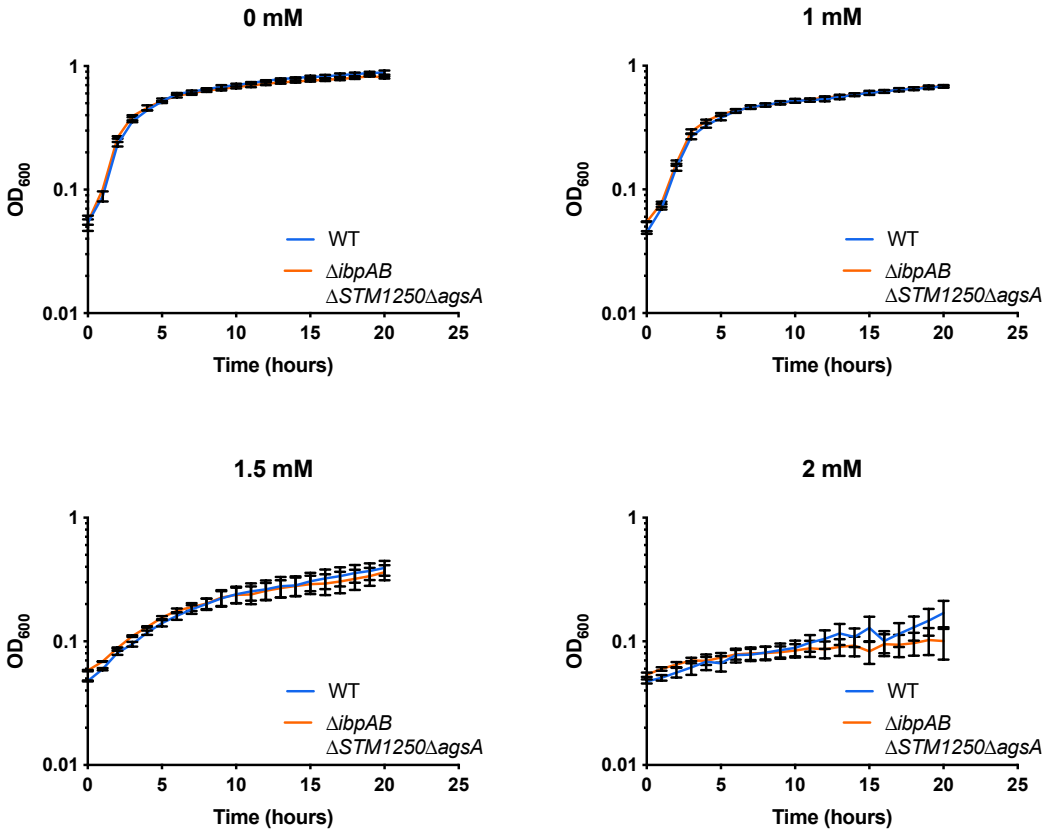


Figure 41. Growth in LB containing increasing concentrations of zinc chloride ($ZnCl_2$). Bacteria were grown in 24-well plates, aerobically in LB containing increasing concentrations of $ZnCl_2$ at 37 °C for 24 hours. OD_{600} readings were taken hourly by a SpectroMax M5 plate reader (Molecular Devices). Data points representative of the means of three separate experiments performed in duplicate. Error bars represent SEM.

In a similar manner to the growth in $CuCl_2$, the overall growth of *Salmonella* was reduced in the presence of $ZnCl_2$ in a concentration dependent manner, with strains growing in 2 mM $ZnCl_2$ producing very little biomass. However, there was no difference observed between the growth of WT or the quadruple mutant in the presence of 1, 1.5 or 2 mM $ZnCl_2$. As a result, the response of the remaining mutants was not tested and it was concluded that IbpAB, STM1250 or AgsA do not contribute to zinc resistance in the conditions tested in this study.

3.2.9 Growth of Δ*ibpAB*Δ*STM1250*Δ*agsA* is not affected by 2,2-dipyridyl

Previous characterisation of the *Salmonella* Fur regulon by Troxell et al. (2011) identified that *STM1250* was upregulated in a Δ*fur* mutant, indicating that in WT it is repressed by Fur. In iron rich conditions, Fur complexes with iron and can prevent transcription through binding to the conserved Fur-box. However, in iron-limiting conditions, iron and Fur do not complex and transcription occurs (Rychlik and Barrow, 2005).

The proposed repression of *STM1250* by Fur suggests that under iron limiting conditions, *STM1250* may be required. In addition, the characterisation of gene expression during stress identified the upregulation of *ibpA* and *ibpB* following low iron shock (Kröger et al., 2013). To investigate the involvement of IbpAB, STM1250 and AgsA in the growth in iron-limiting conditions, the *Salmonella* quadruple mutant was grown in increasing concentrations of the iron chelator 2,2-dipyridyl (2,2-Dip). Concentrations were selected based on those utilised in a previous study of the identification of *Salmonella* iron-dependent gene expression (Karash and Kwon, 2018).

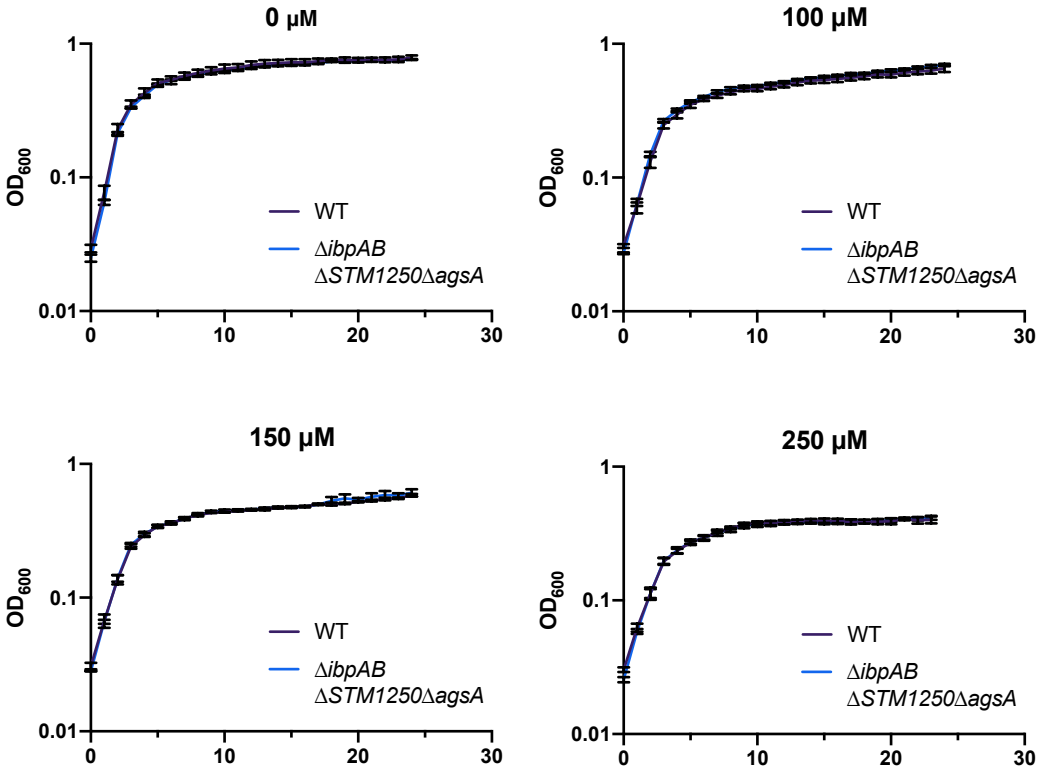


Figure 42. Growth of the quadruple mutant is unaffected by the addition of 2,2-dipyridyl. Bacteria were grown in LB in a 24-well plate containing varying concentrations of the iron chelator 2,2-dipyridyl at 37 °C for 24 hours. OD_{600} measurements were taken hourly for 24 hours by a SpectroMax M5 plate reader (Molecular Devices). Data points are representative of the means of three replicates and error bars represent SEM.

Bacteria were cultured in LB aerobically in a plate reader for 24 hours and OD_{600} readings taken hourly. The overall growth of *Salmonella* was reduced by the addition of 2,2-Dip in a concentration dependent manner (Figure 42). However, no significant difference between growth was observed between WT and the mutants tested in LB containing 2,2-Dip. As a result, susceptibility of the double and triple mutants was not tested.

3.2.10 IbpAB, STM1250 and AgsA are not required for growth in high salt LB

Salt treatment induces a state of cellular osmotic stress. In the food industry, salt is commonly used as a preservative since it disrupts bacterial growth. The ability of pathogens to overcome osmotic stress is key to their persistence in the food chain. Moreover, it has been indicated that osmotic stress induced cross resistance is

possible, leading to increased resistance to host associated stresses during infection, for example acid stress (discussed in more detail in Chapter 4) (Burgess et al., 2016). Bacterial chaperones are upregulated during osmotic stress with an important role in the prevention and reduction of intracellular protein aggregation (Finn et al., 2015). Furthermore, gene expression studies have indicated the upregulation of *ibpA*, *ibpB*, *STM1250* and *agsA* under salt stress (Figure 16) (Kröger et al., 2013, Finn et al., 2015).

In order to investigate the contribution of IbpA, IbpB, STM1250 and AgsA to the ability of *Salmonella* to survive in the presence of high salt concentration and osmotic pressure, bacteria were grown in either standard LB media containing 1% (w/v) NaCl or high salt LB media containing 6% (w/v) NaCl and were incubated at 37 °C for 24 hours with OD_{600} readings taken hourly.

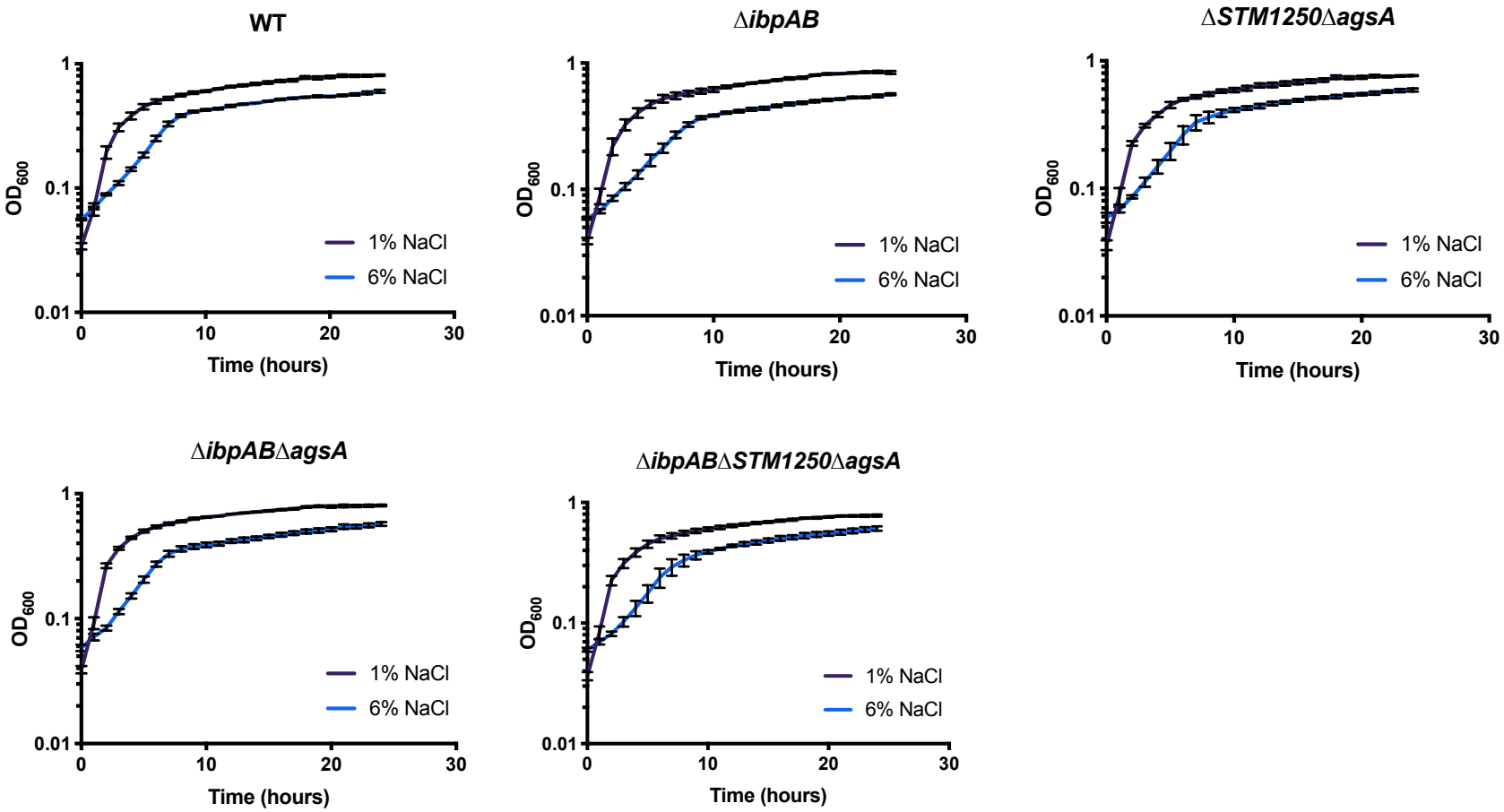


Figure 43. Growth on high salt does not affect overall growth of WT or mutant strains. Bacteria were grown in a 24-well plate in LB containing either 1% (w/v) or 6% (w/v) sodium chloride at 37 °C for 24 hours. OD_{600} readings were taken hourly by a SpectroMax M5 plate reader (Molecular Devices). Data points representative of the means of three separate experiments performed in duplicate. Error bars represent SEM.

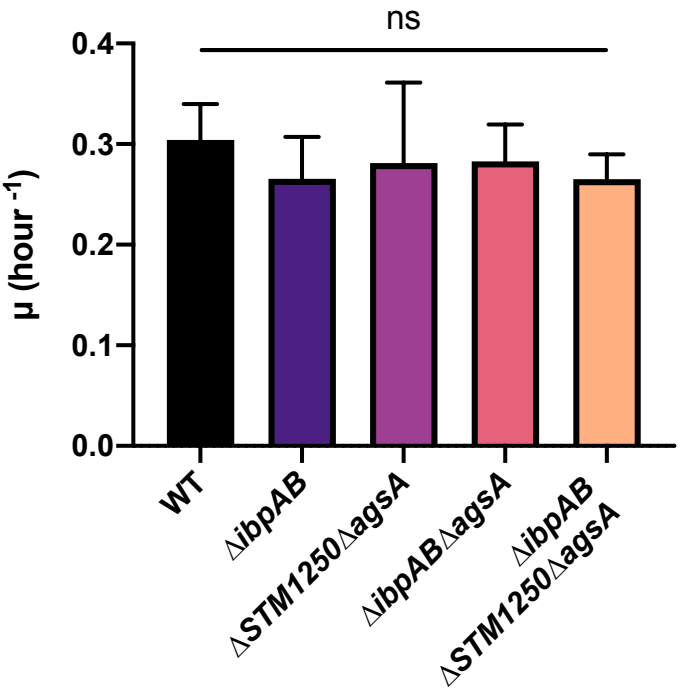


Figure 44. *ibpA, ibpB, STM1250* or *agsA* do not contribute to the initial growth rate (μ hour^{-1}) of *S.* Typhimurium during growth in high salt LB. Bacteria were grown in 24-well plates in LB containing 6% (w/v) NaCl at 37 °C and OD_{600} readings were taken hourly. Data shows the means of three separate experiments performed in duplicate. Error bars are SEM. Data was analysed by one-way ANOVA with Tukey's post-test. ns $p > 0.05$ vs WT.

Growth in 6% (w/v) NaCl LB increased the lag phase of all *Salmonella* strains tested, compared against growth in 1% (w/v) NaCl LB (Figure 43). Despite this, calculation of the initial growth rate did not identify significant differences between the WT and mutant strains (Figure 44).

3.2.11 The quadruple mutant shows increased sensitivity to polymyxin B but is not sensitive to colistin

Resistance to cationic antimicrobial peptides is of significant importance to enteric pathogens. During an infection, host intestinal immune cells release cationic AMPs as part of the immune response to kill invading bacteria (Diamond et al., 2009). Exposure to host-derived cationic AMPs has been shown to induce the expression of *Salmonella* virulence genes during infection (Bader et al., 2003). In addition, the polymyxin cationic AMPs are among the last-line antimicrobials used in the treatment

of many multiple drug resistant Gram-negative infections. Polymyxin B is a cationic AMP, which binds to the negative charge of Gram-negative LPS. Interactions with LPS disrupts OM homeostasis and can cause cell lysis. Polymyxin B has been used previously in investigations of bacterial OM integrity (Appia-Ayme et al., 2012). In order to test sensitivity to polymyxin B, cell killing assays and minimum inhibitory concentration (MIC) assays were performed. In cell killing assays, exponential phase bacteria were challenged with 2 µg/mL polymyxin B for one hour. Bacterial CFU/mL were determined for pre- and post-challenged cells for the calculation of overall percentage survival (Figure 45).

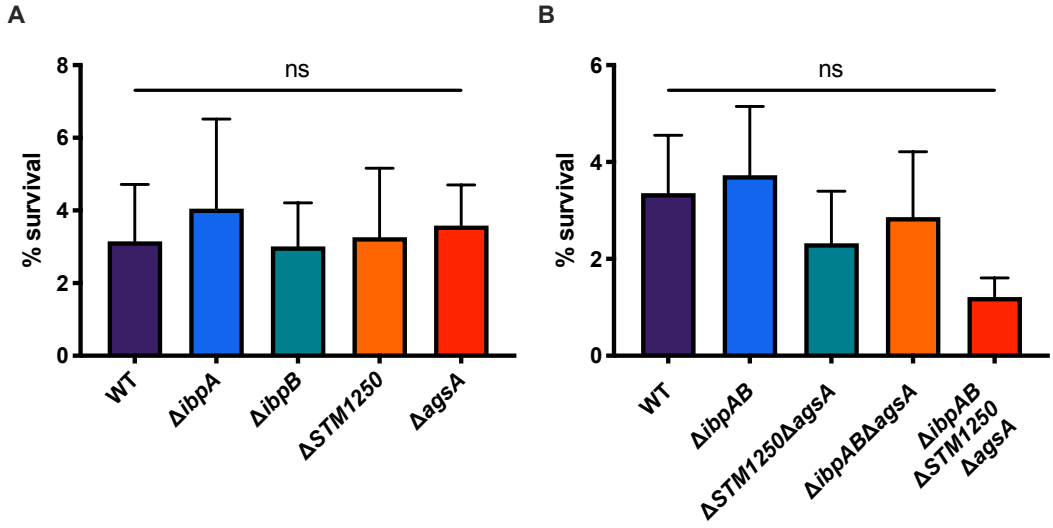


Figure 45. Single *ibpA*, *ibpB*, *STM1250* and *agsA* deletions do not affect survival but survival of the quadruple mutant is reduced following treatment with 2 µg/mL polymyxin B. Single deletion mutants (A) and multiple gene deletion mutants (B). Bacteria were grown to OD_{600} 0.1 before the addition of 2 µg/mL polymyxin B. Bacteria were incubated at 37 °C for 1 hour with polymyxin B. Data points are representative of four separate experiments performed in duplicate and error bars represent SEM.

Cell killing assays demonstrated an overall trend in reduction of survival of the quadruple mutant, and in addition the average percentage survival of the Δ*STM1250*Δ*agsA* double mutant and the Δ*ibpAB*Δ*agsA* triple mutant were mildly reduced compared to WT. However, due to variability across all samples, statistical analysis (one-way ANOVA) concluded that the reductions in survival were not significantly different in comparison to the WT.

Minimum inhibitory concentration (MIC) assays were performed in 96-well plates with increasing concentrations of polymyxin B and each well was inoculated with a standardised bacterial culture. Overall, the quadruple mutant had a lower MIC range than the WT or the remaining mutant strains. At 4 µg/mL and 2 µg/mL there was a significant reduction in the final OD_{600} of the quadruple mutant in comparison to the WT. Interestingly, all mutant strains were reduced at 4 µg/mL but only the quadruple mutant was statistically different (one-way ANOVA, $p < 0.05$).

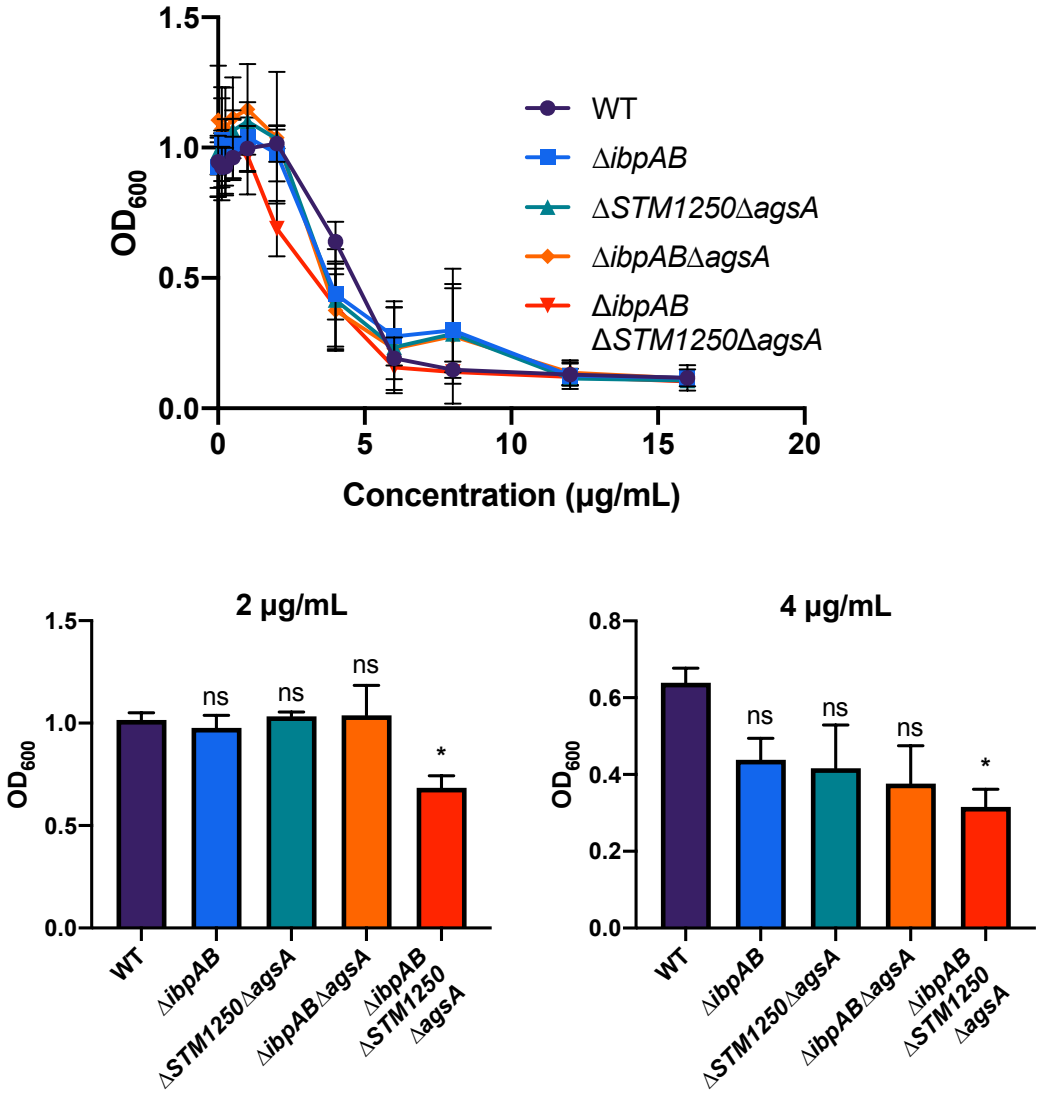


Figure 46. The combined deletion of *ibpA*, *ibpB*, *STM1250* and *agsA* increases sensitivity of *Salmonella* to 4 µg/mL and 2 µg/mL polymyxin B. Minimum inhibitory concentration assays were performed in 96-well plates containing increasing concentrations of polymyxin B. At 4 µg/mL and 2 µg/mL the resulting OD_{600} of the quadruple mutant was significantly lower than that of WT. Data are the means of three separate experiments performed in quadruplicate. Data was analysed by one-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$ vs. WT.

Colistin (polymyxin E) is also a cationic AMP in the polymyxin B family. Despite their highly similar mechanisms of action and toxicity the two drugs differ in their structures by only a single amino acid. However their potency and dosage in patients differs; whilst both drugs cause the displacement of Ca^{2+} and Mg^{2+} from the LPS molecules,

colistin can also bind to and neutralise the lipid A component of LPS, thereby increasing its toxicity (Gupta et al., 2009).

In the same manner as for polymyxin B, cell killing and MIC assays were performed. For cell killing assays, bacteria were grown to OD_{600} 0.1 before the addition of either 2 µg/mL or 4 µg/mL colistin for 1 hour at 37 °C (Figure 47). In contrast to polymyxin B treatment, the percentage survival of both WT and the quadruple mutant were low in both treatment conditions, for example following a 2 µg/mL challenge, only 1% of WT were remaining, a four-fold reduction in comparison to 2 µg/mL polymyxin B treatment. These data supported the fact that colistin is more toxic than polymyxin B. Despite this, there was no significant reduction in survival of the quadruple mutant compared to the WT in either condition.

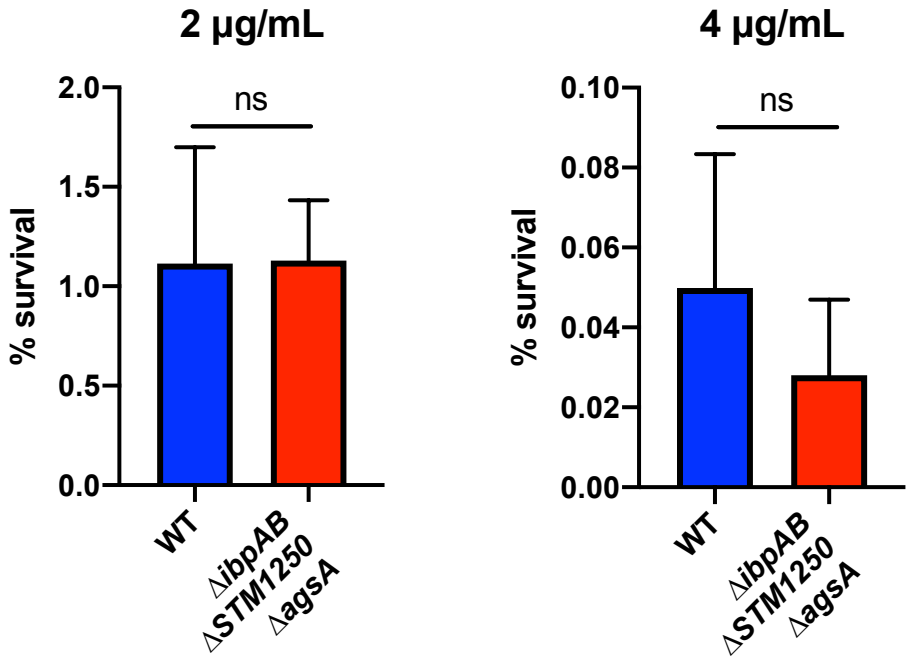


Figure 47. The survival of the quadruple mutant is reduced following treatment with 4 µg/mL colistin. Bacteria were grown to OD600 0.1 before the addition of 2 µg/mL or 4 µg/mL colistin. Bacteria were incubated at 37 °C for 1 hour with colistin. Data points are representative of three separate experiments performed in duplicate and error bars represent SEM.

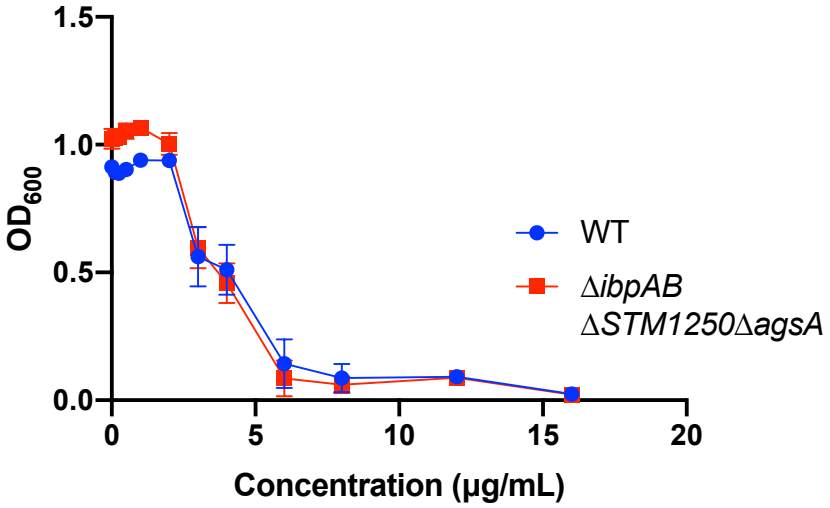


Figure 48. The combined deletion of *ibpA*, *ibpB*, *STM1250* and *agsA* does not increase sensitivity of *Salmonella* to colistin. Minimum inhibitory concentration assays were performed in 96-well plates containing increasing concentrations of colistin. Data are the means of three separate experiments performed in quadruplicate.

In support of the cell killing assays, MIC analysis demonstrated that there were no significant reductions in sensitivity of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant compared to WT across the concentration ranges tested. In fact, in the lower concentration range of 0 µg/mL to 2 µg/mL, the quadruple mutant was more resistant to colistin.

Since cationic antimicrobial peptides elicit their bactericidal effects via primarily interacting with the LPS present on the outer leaflet of the Gram-negative OM, we investigated the composition of the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* LPS. LPS were extracted from O/N cultures and separated by SDS-PAGE before visualisation by silver staining.

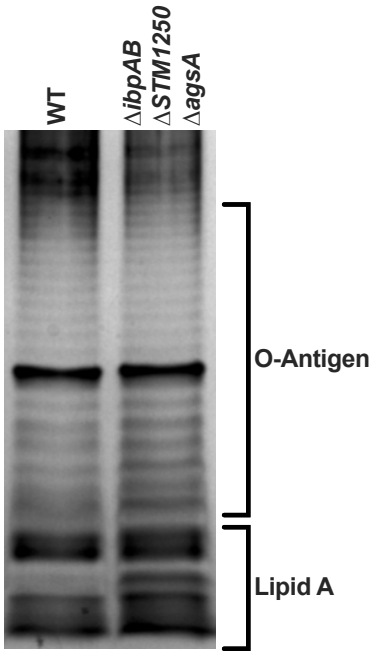


Figure 49. LPS silver stain analysis may reveal differences between WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* lipid A cores. Bacterial LPS were isolated from O/N cultures and run on SurePAGE™ 4-20% Bis-Tris pre-cast SDS-PAGE gels (GenScript). LPS was detected by silver stain using the SilverQuest™ Silver Staining Kit (Invitrogen). Gel image is representative of 3 separate LPS extractions.

LPS separation did not reveal any notable differences between the O-antigen or lipid A chain lengths. However, there is an additional band present in the lipid A region of the Δ*ibpAB*Δ*STM1250*Δ*agsA* sample that was only minimally visible in the WT sample (Figure 49) and the implications of this are discussed in 3.3.4.

3.2.12 Deletion of *ibpAB*, *STM1250* and *agsA* does not increase *Salmonella* sensitivity to vancomycin

Although the proteins of interest in this study are predicted to be cytoplasmic, previous reports have described the localisation of IbpA in the OM fraction of *E. coli* following heat shock (Laskowska et al., 1996). Coupled to the findings in this chapter, that the survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is reduced under heat and cold stress, in addition to the sensitivity to polymyxin B, we tested whether the proteins may be involved in the maintenance of overall OM integrity by measuring the survival of the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* strains to vancomycin. Gram-negative bacteria are intrinsically resistant to the antibiotic vancomycin, which is unable to pass

the cell envelope. However, when OM integrity is significantly compromised, vancomycin is highly toxic towards Gram-negative bacteria (Justice et al., 2005).

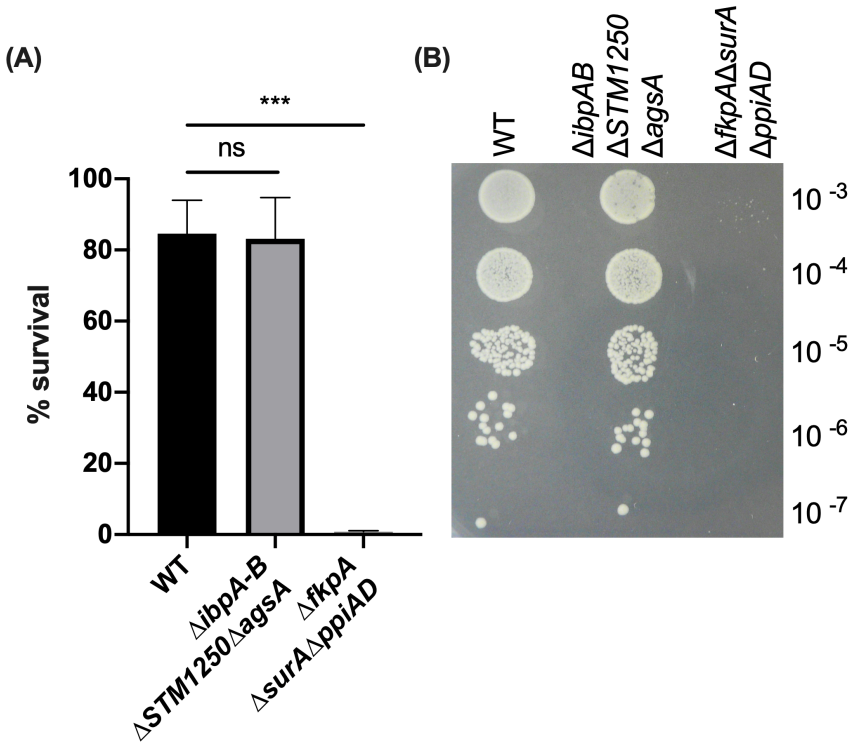


Figure 50. The Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is not sensitive to 65 µg/mL vancomycin. O/N cultures were serially diluted in PBS and spotted (10 µL) onto LB agar containing 65 µg/mL vancomycin. The quadruple mutant Δ*fkpA*Δ*surA*Δ*ppiAD* was used as a control to confirm efficacy of the antibiotic. (A) Percentage survival of *Salmonella* strains grown on vancomycin LB agar, calculated relative to LB agar only controls. (B) A representative image of bacterial growth on 65 µg/mL vancomycin. Mean percentage survival was calculated from three separate experiments performed in duplicate and error bars show SEM. Data analysed by one-way ANOVA with Tukey's post-test, *** $p < 0.0001$ and ns $p > 0.05$.

The CFU/mL of bacteria grown on LB agar containing 65 µg/mL vancomycin were compared to that of bacteria grown on LB agar only for calculation of percentage survival. The Δ*fkpA*Δ*surA*Δ*ppiAD* mutant, previously shown to be highly sensitive to vancomycin was used as a control to verify the efficacy of the antibiotic under the experimental conditions in the present study (Justice et al., 2005). Overall, there was no difference between the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant, with both strains

being highly resistant to vancomycin with an 80% survival rate. These data suggest that no substantial overall alteration to the permeability or integrity of the OM barrier is caused by the loss of these stress responsive proteins.

3.3 Discussion

The aim of this chapter was to investigate the contribution of IbpA, IbpB, STM1250 and AgsA to the *Salmonella* ESR. It was hypothesised that in addition to their roles as sHsps, IbpA, IbpB and AgsA are involved in the protection against other extracytoplasmic stresses, in cooperation with the putative stress responsive protein STM1250. In order to address this hypothesis a range of mutants, including a quadruple deletion mutant (Δ*ibpAB*Δ*STM1250*Δ*agsA*) were generated and subjected to a variety of stresses which disrupt OM and envelope homeostasis.

3.3.1 *Salmonella* specific STM1250 and AgsA are conserved differently among localised and systemic serovars

Amino acid sequence analysis of STM1250 and AgsA revealed interesting differences in the conservation of these *Salmonella* specific proteins among *Salmonella* serovars. The STM1250 protein sequences of the serovars *S.* Enteritidis, *S.* Choleraesuis, *S.* Typhi, *S.* Paratyphi and *S.* Dublin and the iNTS strain *S.* Typhimurium D23580 were aligned to the *S.* Typhimurium SL1344 strain. *S.* Enteritidis is commonly associated with foodborne infections, particularly those arising from consumption of eggs (Gantois et al., 2009). *S.* Typhi and Paratyphi are responsible for the human host-restricted infections typhoid and paratyphoid, respectively, as described in Chapter 1. *S.* Dublin is commonly associated with bovine infection where it is host-adapted and causes an enteric fever. However, *S.* Dublin is not host-restricted and infections also occur in swine, sheep and horses. Human infections have occurred via the consumption of raw milk from infected cattle (McDonough et al., 1999). Finally, *S.* Choleraesuis causes a systemic infection in swine and is a NTS serovar with the greatest risk of leading to bacteraemia in humans (Jones et al., 2008).

The α-crystallin sHsp AgsA was present in all serovars investigated and all sequences shared 99-100% homology to SL1344. In contrast, different levels of conservation were observed for STM1250 between the serovars investigated in this study. Both of the *S.* Typhimurium (SL1344 and D23580) sequences shared 100% amino acid homology and *S.* Enteritidis STM1250 was also found to share close homology to that of *S.* Typhimurium SL1344. These serovars cause similar gastroenteritis disease types in humans and other animals. Interestingly, *S.* Typhi was the only enteric fever associated serovar to share a close homology to *S.* Typhimurium STM1250. In comparison, the remaining serovars with the lowest

homology to *S.* Typhimurium SL1344 (Paratyphi, Choleraesuis, Newport and Dublin), all shared almost identical homology to one another. Since STM1250 has not been studied in these serovars and limited genome-wide screens have been published (see below), it is unclear whether the *STM1250* is functional in the *S.* Paratyphi, *S.* Choleraesuis, *S.* Newport and *S.* Dublin serovars.

Further phylogenetic analyses of the STM1250 protein sequence revealed divergence of STM1250. Our analyses suggested that the variations in the STM1250 sequences analysed may correspond to the *Salmonella* serovar phylogenetic lineages. For example, close relationships between *S.* Paratyphi C and *S.* Choleraesuis have been reported (Timme et al., 2013), and the phylogenetic tree reported in this thesis suggests that the STM1250 protein sequence in these serovars is indeed closely related.

The alignments point towards multiple possible observations. Firstly, that STM1250 is not associated to a specific disease type, be it localised or systemic infection as it is present in serovars causing both types of disease. Secondly, that STM1250 has been acquired following divergence of the *S. enterica* species due to its absence in *S. bongori*, strongly suggesting that it is involved during host infection. The development of advanced screening technologies such as transposon site hybridisation (TraSH), transposon mediated differential hybridisation (TMDH) and transposon directed insertion sequencing (TraDIS) have allowed for high-throughput identification of genes required for fitness of *Salmonella* in a range of hosts. Previous studies have indicated that *STM1250* mutants are not significantly attenuated in mice (Lawley et al., 2006, Santiviago et al., 2009, Chaudhuri et al., 2009) and *agsA* but not *STM1250* mutants are attenuated in cattle (Chaudhuri et al., 2013). Although the investigations in this thesis have only considered STM1250 in *S.* Typhimurium SL1344, future work should expand investigations into the role of STM1250 in the stress responses and physiology of other serovars. Of particular interest would be the human host-restricted *S.* Typhi and the iNTS variant of *S.* Typhimurium, both of which exhibit invasive disease manifestations but which are not modelled exactly *in vivo* due to the lack of an experimental host.

3.3.2 The IbpA, IbpB and AgsA response to heat and cold shock

Heat stress has a highly adverse effect on numerous cellular processes, therefore a counter-response and the ability to repair damaged structures is essential for bacterial survival. As outlined above, extreme temperatures increase membrane fluidity, induce the release of LPS from the cell surface and have an adverse effect

on overall cell integrity (Denich et al., 2003). Heat shock induces the expression of a multitude of chaperones and proteases, collectively described as the Hsps and sHsps. Gram-negative bacteria possess a core set of Hsps which are members of the chaperone family, including IbpA, IbpB DnaK, DnaJ, GrpE, GroEL and GroES. As well as chaperones, heat shock induces the expression of proteases which are important for the degradation of damaged proteins that are beyond repair with examples including Lon and HtrA (DegP) (Arsène et al., 2000).

Expression of the IbpAB and AgsA sHsps are controlled by unique 5' UTR RNA thermometers, controlling their transcription in a temperature dependent manner (Waldminghaus et al., 2007, Gaubig et al., 2011). At 37 °C, RNA thermometers are sufficiently melted to enable gene expression. However, expression significantly increases at temperatures in excess of 42 °C (Narberhaus et al., 2006). To ensure that the genes were being expressed in the WT under heat shock conditions, in this study we investigated the response of *Salmonella* to heat stress at 50 °C. Previous studies of the *Salmonella* heat shock response have identified AgsA, a *Salmonella* specific sHsp containing the α-crystallin domain (Tomoyasu et al., 2003). Previously, an *agsA* deletion alone did not affect *Salmonella* survival at the extreme temperature of 70 °C (Tomoyasu et al., 2003). However, in the same study, an Δ*ibpAB*Δ*agsA* triple deletion mutant showed reduced survival at this extreme temperature. Moreover, Tomoyasu et al. (2003) confirmed expression of AgsA in a *Salmonella* Δ*ibpAB* mutant, suggesting that it compensates for the loss of these two sHsps. Interestingly, despite lacking the *Salmonella* specific AgsA sHsp, *E. coli* Δ*ibpAB* mutants are unaffected by 50 °C heat shock (Thomas and Baneyx, 1998).

In this study, after a 2-hour incubation at 50 °C the survival of all mutants tested (Δ*ibpAB*, Δ*STM1250*Δ*agsA*, Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA*) was unchanged compared to WT. However, after 4 hours, our data agreed with previous published literature (Thomas and Baneyx, 1998, Tomoyasu et al., 2003) in that the single and double mutants (Δ*ibpAB* and Δ*STM1250*Δ*agsA*) were unaffected at 50 °C but survival of the triple mutant was significantly reduced, compared to WT. Previous studies have focussed on the short-term heat shock response (Tomoyasu et al., 2003). However, the findings in this study also suggest a role for the sHsps in the survival of *Salmonella* during prolonged heat stress. This may be explained by a potential increase of protein damage and aggregation over the 4 hour incubation period which requires the continued expression of the sHsps.

Under the conditions tested in this study, there was no difference between survival of the triple and quadruple mutants suggesting that STM1250 does not function as a

sHsp. Although STM1250 has not been annotated in the literature as a member of the sHsp family due to its lack of an α-crystallin domain, it has been shown to be upregulated during recovery of *Salmonella* from heat stress at 55 °C (Hsu-Ming et al., 2012). Despite this, in this thesis the data do not indicate that STM1250 is required for survival in extreme temperatures. The previously reported upregulation of STM1250 during heat stress recovery (Hsu-Ming et al., 2012) could simply be due to the induction of *rpoE* and subsequent upregulation of STM1250. Alternatively, STM1250 may have a specific role in heat stress management, specifically during recovery as opposed to when the stress is still present, that has yet to be identified.

Despite the reduced survival of the mutants lacking *ibpA*, *ibpB* and *agsA*, when growth curves were performed at sustained high temperature (47 °C) there was no notable difference between growth of the WT and mutants strains. These data suggest that the production/requirement of these sHsps is only important following sudden changes in temperature, in the mid-exponential growth phase, or above a certain temperature threshold. Interestingly, a previous study has reported that growth of a *Pseudomonas putida ibpA* mutant is defective at 40 °C compared to WT (Krajewski et al., 2013). This may be explained by the fact that *Pseudomonas* sp. lack the functionally overlapping *ibpB* gene and highlights the importance that these sHsps are studied in a range of Gram-negative bacteria, in addition to the widely studied *E. coli*.

Cold shock is one of the major inducers of *rpoE* in *S.* Typhimurium (Miticka et al., 2003), in contrast this response is not observed in *E. coli* (Dartigalongue et al., 2001). Temperature downshifts lead to a reduction in membrane fluidity, reduce efficiency of transcription and translation and results in error-prone protein folding (Keto-Timonen et al., 2016). The bacterial cold shock response involves an acclimation stage and an adapted stage (Zhang et al., 2018). During the former, cell growth is arrested and protein production, with the exception of cold shock proteins, is halted (Zhang et al., 2018). Since cold shock affects proteins and membranes in a somewhat similar manner to heat shock, it was hypothesised that sHsps may also respond to cold stress. Moreover, a *Salmonella* cold shock protein, CspH (STM1243), is located upstream of STM1250 and AgsA.

In this study, it was shown that following cold shock at 10 °C the WT continued to multiply for the first 30 mins of incubation, whilst during this period, the cold treatment halted the growth of the Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants. These results indicated that loss of the IbpA, IbpB and AgsA proteins increased sensitivity of *Salmonella* to the effects of a 10 °C incubation. In their roles as chaperones and

preventing irreversible protein aggregation, the sHsps may protect the cell from increased errors in protein folding as a result of cold exposure. The lack of difference between the triple and quadruple deletion mutants suggest that STM1250 does not significantly contribute to the prevention of protein aggregation at low temperatures. In order to further elucidate the contributions of these proteins to the bacterial cold shock response, it would be beneficial to determine the expression levels of *ibpA, ibpB, STM1250* and *agsA* under cold conditions by qRT-PCR. Furthermore, since only the triple and quadruple mutants were assessed for their ability to survive cold temperatures, it would be beneficial if future studies also considered the survival of the single mutants (Δ*ibpA,* Δ*ibpB,* Δ*STM1250* and Δ*agsA*)

As a general rule, sHsps are cytoplasmic; however, the potential of temperature-induced damage to the cell envelope indicates an additional importance for heat shock responsive proteins in the cell envelope stress response. In support of this, Laskowska et al. (1996) identified that *E. coli* IbpA and IbpB are localised to the OM following heat shock, indicating a potential role for IbpA and IbpB that extends beyond the maintenance of cytoplasmic homeostasis. To date, it is unclear whether AgsA and STM1250 also contribute to envelope homeostasis and whether IbpAB localise to the OM in *Salmonella*. Coupled to the ideas discussed in Chapter 1, that RpoH tethering at the IM may result in the control of periplasmic and IM processes, there is a suggestion that IbpA and IbpB, and indeed AgsA and STM1250, may influence envelope processes. To this end, in this study phenotypic analyses were extended beyond temperatures-based stress. Further experiments addressed whether IbpA and IbpB, in cooperation with the hypothesised functional partners AgsA and STM1250, may be involved in the protection against additional inducers of envelope stress. In agreement with this hypothesis, various stresses including oxidative stress, metals, salinity and antimicrobials have in fact been shown to induce the prokaryotic 'heat shock response', due to the increased expression of Hsps such as DnaK, GrpE and ClpB (Cardoso et al., 2010, Roncarati and Scarlato, 2017).

3.3.3 IbpA, IbpB, STM1250 and AgsA do not respond to metal-induced or osmotic cell stress in *Salmonella* Typhimurium

A previous study in *E. coli* reported that IbpA/B are important for copper tolerance, specifically to protect against oxidative damage induced by copper ions (Matuszewska et al., 2008). Copper is an essential cofactor for many metabolic and respiratory enzymes, yet excess copper is highly toxic if homeostasis is not maintained (Argüello et al., 2013). Excess intracellular copper can compete with other

metals for enzyme cofactor binding sites, catalysing the formation of non-native disulphide bonds and the formation of reactive oxygen species (ROS) (Kimura and Nishioka, 1997). Copper ions (Cu^{+} and Cu^{2+}) will react with molecular oxygen (O_2), superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2), leading to the formation of hydroxyl radicals (OH^{-}). Therefore, maintenance of intracellular copper homeostasis is critical for bacterial survival.

Our understanding of bacterial copper homeostasis, as is the case for many stress response mechanisms, is derived mainly from work in *E. coli*. Homeostasis is maintained primarily via the efflux of excess copper via the Cue (Cu efflux) system which involves the CopA P-type ATPase copper transporter (Bondarczuk and Piotrowska-Seget, 2013). *E. coli* also possess the CusRS (Cu sensing) system, a TCS regulating the CusCBA transport machinery, involved in the efflux of copper ions from the cytoplasm and into the extracellular space (Munson et al., 2000). In terms of the effect of copper on envelope homeostasis, multiple ESRs including σ^E (Egler et al., 2005) and CpxAR (Yamamoto and Ishihama, 2006) are induced under high copper stress and are arguably as important for bacterial survival as the Cue and Cus homeostatic systems.

In *E. coli,* IbpA and IbpB are predicted to be part of a second line of defence, required when intracellular copper levels are too high and beyond the usual homeostatic control of the efflux and ESR systems (Matuszewska et al., 2008). In fact, *E. coli* Δ*ibpAB* mutants have been shown to exhibit increased sensitivity to copper during aerobic growth (Matuszewska et al., 2008). The authors of the previous study identified that increased copper sensitivity was linked to the production of ROS, as opposed to an inability of the bacterium to expel excess intracellular copper. Interestingly, Ladomersky and Petris (2015) reported that copper-medicated -OH production is confined to the periplasm in *E. coli* and this supports the hypothesis in this thesis that the role of the IbpAB proteins extends beyond the cytoplasm and into envelope maintenance processes.

The contributions of IbpA/B to *Salmonella* copper resistance have, to date, not been studied. In this thesis growth curve assays in LB containing copper demonstrated that the *Salmonella* Δ*ibpAB* mutant was not sensitive to copper. Continuing on the theme of functional redundancy we hypothesised that this may be due to functional overlap with the STM1250 and AgsA proteins, which are not present in *E. coli*. However, as for Δ*ibpAB,* no difference between the WT and the Δ*STM1250*Δ*agsA* or Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants was observed during growth of *Salmonella* in the presence of 2 mM, 3 mM or 4 mM $CuCl_2$. Sensitivity of *Salmonella* was also assessed

during growth on LB agar containing 4 mM $CuCl_2$, a method that has previously associated the Cpx response with copper resistance (Wells, 2015). In agreement with the growth curve assays, no difference was observed between WT and Δ*ibpAB*Δ*STM1250*Δ*agsA*. These data suggest that unlike *E. coli*, in *Salmonella* these proteins are not required for resistance to copper or indeed copper-induced oxidative stress. The lack of phenotype for the mutants in this study may be due to the fact that *Salmonella* copper detoxification systems differ to *E. coli*. The *cue* system is conserved in *Salmonella*, but the *cus* system is not present (Pontel and Soncini, 2009). *Salmonella* also possesses a *cue*-like system called *gol,* although a role for this system in copper tolerance has only been shown in *Salmonella* Δ*copA* mutants (its primary role is in resistance to gold salts) (Espariz et al., 2007). Furthermore, *Salmonella* has been shown to tolerate higher copper concentrations than *E. coli*, particularly in anaerobic conditions (Pontel and Soncini, 2009).

The difference between the capabilities of *Salmonella* and *E. coli* copper tolerance was further demonstrated by a side-by-side comparison of the growth an SL1344 and *E. coli* K12 Δ*ibpAB* mutant in the presence of 3 mM and 4 mM copper. While the *Salmonella* WT and Δ*ibpAB* mutant in the presence of 3 mM or 4 mM $CuCl_2$ grew almost equally to the untreated control, the *E. coli* WT and Δ*ibpAB* strains were both significantly affected by the 4 mM treatment, with overall biomass almost 3-fold lower compared to the untreated control. Interestingly although it has been previously reported that an *E. coli* Δ*ibpAB* mutant is sensitive to 3 mM copper, we did not observe a statistically significant difference between the final OD_{600} of the *E. coli* WT and Δ*ibpAB* mutant. The experiment was performed as described by Matuszewska et al. (2008) so the reasons for these differences is unclear. However, it should be noted that statistical analysis was not performed on the data reported in the Matuszewska et al. (2008) study. Furthermore, in the study performed by Matuszewska et al. (2008) the *E. coli* cultures were incubated at 30 °C. The reasons for this choice of temperature are unclear, especially since *ibpA* and *ibpB* are controlled by a temperature sensitive RNA thermometer and expression of both genes is predicted to be low below 37 °C. It may be beneficial, therefore, if future studies are performed at elevated temperatures. Indeed, such studies may reveal a temperature-associated response to copper.

Overall, the investigations in this thesis indicated that IbpA, IbpB, STM1250 and AgsA are not involved in *Salmonella* resistance to copper. This was supported by our initial data that alluded to the fact that overexpression of STM1250 or AgsA in an *E. coli* Δ*ibpAB* mutant background does not increase survival in the presence of copper.

However, limited conclusions can be drawn from these data and further work should be performed using different concentrations of IPTG for overexpression of STM1250 and/or AgsA. In this study, 1 mM IPTG was used and the increased level of protein expression, coupled with exposure to copper stress conditions, may have affected bacterial growth.

The proteins in this study have been implicated in the resistance to other metals and copper is certainly not the only metal that, in excess, is toxic to bacteria. Previously, heterologous overexpression of AgsA in *E. coli* has been shown to increase the EC_{50} of *E. coli* to cadmium and zinc (Ezemaduka et al., 2018, Lv et al., 2019). Zinc metalloproteins form a between 5 and 6% of the bacterial proteome (Capdevila et al., 2016) and maintenance of intracellular free zinc, which is kept at picomolar to femtomolar concentrations (Blindauer, 2015), is extremely important for bacterial homeostasis and survival. Previous studies have identified a zinc responsive TCS, ZraSR, and IbpA and IbpB have been shown to be upregulated on its overexpression (Wells, 2015). These data implicated a potential role for the proteins in this study for survival in high zinc environments. The WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant were grown in the presence of 0 mM, 1 mM, 1.5 mM and 2 mM $ZnCl_2$. Overall, the growth of both strains was reduced in a concentration dependent manner but there was no difference between the WT and mutant in each condition. The lack of phenotypic difference between WT and mutant indicates that zinc homeostasis mechanisms are able to function as usual and tolerate the increased environmental zinc concentrations.

Due to the importance of metals as protein cofactors, metal limiting conditions are also important inducers of bacterial stress responses. The Fur protein is one of the major controllers of iron homeostasis in *Salmonella* and under iron-rich conditions, complexes with iron and prevents gene expression. When iron concentrations are low, iron is titrated away from Fur, releasing its repression of gene expression (Rychlik and Barrow, 2005). Troxell et al. (2011) reported that *STM1250* is upregulated in a Δ*fur* mutant suggesting that *STM1250* is upregulated under iron starvation conditions. Growth curves of the WT and quadruple mutant were performed in the presence of the iron-chelator 2,2-dipyridyl but there was no significant difference observed between these two strains. In a similar manner to the excess metal results, it is likely that the core iron homeostasis mechanisms continue to function in the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant and therefore a significant phenotype is not observed. One limitation of these analyses was the use of LB media. Future work should also include an assessment of the ability of WT and mutant *Salmonella* to

grow in the presence of elevated copper and zinc, and in limited iron conditions, in M9 minimal media.

Finally, the ability of the WT and mutant strains to grow in the presence of increased salinity were assessed. Growth curve assays in LB containing 6% salt revealed a reduced ability of all strains to grow in comparison to that in LB containing 1% salt. However, analysis of the initial growth rates did not reveal any significant sensitivities of the mutant strains, compared to the WT in 6% salt LB. In future studies, the survival of the mutants in mid-exponential phase to osmotic shock may reveal more significant roles.

3.3.4 The Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is sensitive to polymyxin B, but not colistin or vancomycin

The polymyxin family of antibiotics includes polymyxin B and colistin (polymyxin E). Although the use of polymyxins in the clinic has been extremely limited in previous decades due to their renal toxicity, the dramatic increase in global antimicrobial resistance has led to the increase in their use in recent years (Zavascki and Nation, 2017, Vaara, 2019). Now, the polymyxins are among the last-resort antibiotics used against Gram-negative infections. As such, understanding the mechanisms of resistance to this family of antimicrobials is of vital importance should their use in the clinic and for the treatment of MDR infections be continued. Polymyxins act specifically on Gram-negative bacteria via interactions with the LPS on the OM. With a positive charge, the polymyxins are able to substitute for the Ca^{2+} and Mg^{2+} ions located within the LPS structure, resulting in a detrimental destabilisation of the LPS and OM (Trimble et al., 2016). Surprisingly, the complete mechanisms of action of the polymyxin antibiotics are not entirely understood but the general consensus is that polymyxins target bacterial membranes. Although early studies in *Salmonella* suggested that the killing mechanism involves damage to the cytoplasmic membrane (Teuber, 1974), more recently, in a study performed on *P. aeruginosa* this has been disputed, although the use of different bacterial species does raise the question that the mechanism may differ among different bacteria (Zhang et al., 2000, Trimble et al., 2016). Alternative mechanisms have also been suggested including targeting of cell division machinery, increased intracellular ROS production and binding to *E. coli* ribosomes (Trimble et al., 2016). However, evidence for all of the alternate mechanisms is limited. Perhaps there is not a single mechanism of killing but instead, the various targets contribute and collectively result in cell death. Clearly, further studies are required in order for the precise mechanisms to be understood and further

investigations into the mechanisms of bacterial resistance to polymyxins may aid in this understanding.

Resistance to polymyxins is mediated by the modifications of the LPS, preventing the initial interactions and internalisation of the antimicrobials. In the case of *Salmonella* polymyxin B resistance is acquired and is mediated by the PhoPQ and PmrAB two component systems, resulting in alterations to LPS and an inability of polymyxin B to bind to the cell surface (Gunn and Miller, 1996)). The σ^E regulon also encodes genes involved in cationic AMP resistance (Crouch et al., 2005). In addition to chromosomal encoded resistance mechanisms, reports of AMP resistance arising from horizontal gene transfer has increased in recent years via mobile colistin resistance (*mcr*) genes (Liu et al., 2016).

Deletions of σ^E-regulated periplasmic chaperones have been shown to result in an increase in sensitivity to polymyxin B shock (Appia-Ayme et al., 2012). With the proposed OM localisation of IbpA and IbpB during stress and hypothesised regulation of STM1250 by PhoPQ we investigated the response of the mutants in this study to polymyxin B challenge. In this current study, we observed that survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was reduced following treatment with 2 µg/mL polymyxin B, although one-way ANOVA statistical analysis did not prove this reduction to be statistically significant. However, minimum inhibitory concentration assays highlighted a lower MIC of the quadruple mutant compared to WT and the final OD_{600} of Δ*ibpAB*Δ*STM1250*Δ*agsA* cultures grown in the presence of 2 µg/mL and 4 µg/mL polymyxin B was significantly lower than that of WT. In comparison, the double and triple mutants were not significantly affected, supporting our proposed hypothesis that a level of functional overlap exists between the sHsps and STM1250 under certain extracytoplasmic stresses.

As described above, in order to elicit their bactericidal effects, the polymyxins must be able to interact with the LPS; however, the PhoPQ and PmrAB systems are important in the ability of *Salmonella* to make crucial adaptations to the LPS structure and thus resist polymyxin binding. We investigated the composition of the LPS of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant in order to determine whether its reduced survival following polymyxin B shock and reduced MIC were due to improper LPS formation. Unfortunately, the results were inconclusive. Overall, there were no detectable differences in the lengths of the O-antigen chain compared to WT. In addition, the overall composition of the lipid A region was highly similar, although an additional band was present in this region of the Δ*ibpAB*Δ*STM1250*Δ*agsA* sample that was not present in the WT. At this stage, it was not possible to determine whether this

additional structure in the lipid A core was the cause of the polymyxin B sensitivity. Follow-up experiments are certainly required and the intermediate mutants (Δ*ibpAB* Δ*STM1250*Δ*agsA* and Δ*ibpAB*Δ*agsA*) need to be tested. By analysing the LPS composition of these strains, this may elucidate the mechanisms behind the sensitivity of the Δ*ibpAB*Δ*STM1250*Δ*agsA* strain to polymyxin B. Furthermore, mass spectrometry should be performed on LPS purified from WT and mutant strains (treated and un-treated) to more accurately and quantitatively determine any differences between LPS molecules. Finally, it would be interesting to assess the expression of the core LPS synthesis components at both the mRNA and protein level in the WT and mutants. Since the sHsps are known chaperones and STM1250 is a predicted stress response protein, potential changes in the protein levels of the LPS synthesis machinery may provide insight into the targets of the stress response proteins in this study.

Interestingly, the same response was not observed for colistin. Also known as polymyxin E, colistin is a member of the cationic AMP polymyxin family with a similar mechanism of action to polymyxin B. It is unclear why the response of the mutants in this study differed between these two antimicrobials and this certainly warrants further study and may provide important insights into the mechanism behind IbpAB, STM1250 and AgsA protection against polymyxin B. Although the mechanism of action and mechanism of bacterial resistance for both drugs is extremely similar, colistin sulphate has been shown to possess greater activity towards *Salmonella* species (in addition to *P. aeruginosa* and *Shigella* species) (Falagas et al., 2005). Therefore, this may account for the overall increased rate of killing of *Salmonella* by colistin in this study, and the lack of phenotypic differences observed between WT and mutants. The proposed regulation of *STM1250* by PhoPQ also requires further investigation and this is discussed in more detail in Chapter 4.

Findings in the current and in previous studies have indicated that although the proteins are predicted to be located within the cytoplasm, there are indications that they are important in roles beyond this cellular compartment and may indeed be located within the periplasm or OM sub-compartments during stress. In this study, we utilised vancomycin in order to investigate the OM integrity and permeability of the quadruple mutant. The survival of the mutant was unchanged compared to WT in the presence of vancomycin, indicating that the proteins are not important to overall OM integrity, at least when additional stresses are not present. It is important to note that cold stress has been shown to increase the susceptibility of *E. coli* to glycopeptide antibiotics including vancomycin, through the reduction of outer membrane integrity

(Stokes et al., 2016). This raises the question of whether the mutants in this study would be susceptible to the antibiotics tested when combined with a heat or cold shock treatment. This would be an interesting avenue of future research to elucidate the involvement of the sHsps and STM1250 to the maintenance of OM integrity.

3.3.5 Summary

In this chapter we have identified that, in agreement with previous studies in *E. coli* and *Salmonella*, the sHsps IbpA, IbpB and AgsA are functionally redundant in their ability to protect bacteria against heat stress, and only when all three sHsps are deleted is a phenotype observed. The hypothesis that these sHsps function to protect *Salmonella* against other stresses, not limited to heat, and that functional redundancy exists with the putative stress response protein STM1250 was investigated in an initial broad phenotypic screen.

Although a relationship between IbpAB and copper resistance has been suggested in *E. coli*, this was not observed in *Salmonella*, nor were STM1250 and AgsA linked to metal resistance. However, we have shown for the first time that functional overlap may exist between the proteins in the presence of the cationic AMP polymyxin B. Interestingly this did not extend to the closely related cAMP colistin.

The specificity of STM1250 and AgsA to *Salmonella* is intriguing. The following chapters will probe possible reasons as to why *Salmonella* possess these proteins by investigating their role in environments specific to *Salmonella*, such as those encountered during infection of a host and in the food chain.

Chapter 4 *Salmonella* resistance to host-associated stresses: the contribution of IbpA, IbpB, AgsA and STM1250

Work in this chapter contributed to the following article:

Hews, C. L., Pritchard, E. J. and Rowley, G., 2019. The *Salmonella* Specific, σ^E-Regulated, STM1250 and AgsA, Function With the sHsps IbpA and IbpB, to Counter Oxidative Stress and Survive Macrophage Killing. *Frontiers in cellular and infection microbiology*, 9(263).

4.1 Introduction

In Chapter 3, a broad phenotypic screen was performed in order to investigate the contribution of the sHsps IbpA, IbpB and AgsA and the putative stress responsive protein STM1250 to the protection of *Salmonella* against inducers of envelope stress. During this screen, a novel role for these proteins was identified; the quadruple deletion strain (Δ*ibpAB*Δ*STM1250*Δ*agsA*) was significantly more sensitive to the cationic AMP, polymyxin B, compared to WT *Salmonella*. In addition, sequence analysis of STM1250 across different *Salmonella* serovars revealed its association with the *enterica* species and with serovars that cause both localised and systemic disease, in a variety of hosts.

In order to advance these initial findings and, more specifically, investigate the potential roles of these proteins during infection, this chapter focusses on the survival of *Salmonella* against host-associated stresses, in particular acidic pH, reactive oxygen species and the intramacrophage environment. As indicated in Chapter 3, *ibpAB*, *STM1250* and *agsA* are upregulated under acid stress (Cao et al., 2019) and the sHsps and STM1250 are upregulated during macrophage infection (Eriksson et al., 2003, Canals et al., 2019).

Passage through the host involves multiple key stages, each of which present a myriad of stresses to an invading pathogen. These stresses can include low pH, antimicrobial compounds, reactive oxygen species (ROS) and reactive nitrogen species (RNS), all of which are factors utilised by the host immune system, with the overarching aim of eliminating invading pathogens. An introduction to *Salmonella* pathogenesis and host survival was presented in Chapter 1. Here, a more detailed introduction to the mechanisms of *Salmonella* survival against acid and oxidative stress is provided for context of the results presented in this chapter.

4.1.1 The Salmonella acid tolerance response

As a foodborne pathogen, *Salmonella* is capable of surviving in naturally acidic foods and on food products treated with acid for preservation, such as poultry, while *Salmonella* has also been shown to survive in cheeses, fruit juices and during milk fermentation processes (Alvarez-Ordonez et al., 2009, Álvarez-Ordóñez et al., 2012). The acidic environment of the stomach is one of the first lines of defence against infection (Álvarez-Ordóñez et al., 2011) and is maintained at pH 3, although immediately after eating can reach extremely acidic conditions as low as pH 1.8 (Audia and Foster, 2003). Gastric fluid is comprised primarily of hydrochloric acid

(HCl), but the stomach can also contain organic acids including lactic, propionic, acetic and butyric acids (Álvarez-Ordóñez et al., 2011). HCl within gastric fluid is an important barrier to infection; mice with a mutation in a gastric H^+/K^+ -ATPase proton pump are unable to acidify the stomach, due to an inability to make gastric HCl, and are more susceptible to *Salmonella*, *Yersinia*, *Citrobacter* and *Clostridium* infections in comparison to WT mice (Tennant et al., 2008, Álvarez-Ordóñez et al., 2011). Yet, despite this unfavourable environment, *Salmonellae* are still capable of passing through the stomach, reaching the small intestine and causing infection. In addition to survival in the stomach, once *Salmonella* crosses the epithelial barrier of the intestine and reaches the phagolysosome environment, it must be able to tolerate the acidic SCV which is maintained between pH 4 and pH 5 (Allam et al., 2012). The ability to survive in these hostile environments can be attributed to the acid tolerance response (ATR).

The ATR is an important adaptive mechanism utilised by *Salmonella* that enables survival in extremely acidic conditions. The mechanism, first identified and characterised in *Salmonella* Typhimurium LT2, is defined as the ability to survive acid challenge, in usually lethally low pH conditions (pH 3-4), following adaptation at mild to moderately low pH (pH 4-6) (Foster and Hall, 1990, Foster, 1991, Foster, 1993, Foster, 1995, Foster, 1999). Foster (1991) described two stages of the ATR in exponential phase cells. The pre-shock stage occurs following exposure to pH 5.8 and survival is mediated by pH inducible homeostatic mechanisms (4.1.1.1). Expression of acid shock proteins (ASPs) are induced on exposure to pH 4.3 and together, these two stages increase *Salmonella* survival at pH 3.3. A later study identified that the pre-shock stage is not essential; *Salmonella* is capable of surviving pH 3.3 following a single incubation at pH 4-5 simply due to expression of the ASPs (4.1.1.1) (Foster, 1993).

Although the initial defining works by Foster *et al.*, described the ATR of *Salmonella* in log phase cells, the ATR phenomenon is also observed in stationary phase *Salmonellae* (Lee et al., 1994). In stationary phase cells there are two mechanisms of acid resistance; the first is dependent on pH and provides *Salmonella* with the greatest resistance at pH 4.3 while the other is pH-independent and part of the general stationary phase stress response, dependent on RpoS (Lee et al., 1994, Soo Lee et al., 1995, Foster, 1995). The following sections will describe the different homeostatic and regulatory mechanisms of the ATR.

4.1.1.1 The inducible lysine and arginine decarboxylases

Under non-stressed conditions, the Gram-negative intracellular pH is maintained at pH 7.6-7.8 (Álvarez-Ordóñez et al., 2012). The inducible arginine and lysine decarboxylases play crucial roles in this maintenance of *Salmonella* intracellular pH homeostasis (Park et al., 1996, Alvarez-Ordóñez et al., 2010) and can allow *Salmonella* survival in environments as low as pH 5.8 (Foster, 1991). A glutamate-dependent system has also been observed in *E. coli* and *S. flexneri*, but it is not present in *Salmonella* so will not be discussed further here (Waterman and Small, 2003).

The molecular mechanisms of the lysine and arginine decarboxylase systems in acidic conditions are extremely similar; both systems involve the consumption of an intracellular proton by an amino acid, catalysed by the decarboxylase, followed by export of the resulting compound via a transmembrane antiporter (Viala et al., 2011). The resulting outcome is the reduction of intracellular proton concentration and therefore intracellular pH. The decarboxylase systems have been directly associated with the *Salmonella* ATR and Alvarez-Ordóñez et al. (2010) identified that the supplementation of the adaptation media (pH 4-5) with lysine or arginine increases *Salmonella* acid resistance at the usually lethal pH 2.5 (De Jonge et al., 2003, Alvarez-Ordóñez et al., 2010).

The lysine decarboxylase system is formed of the lysine decarboxylase, CadA, and the lysine-cadaverine antiporter, CadB. The system is induced and transcriptionally activated by the CadC transcriptional regulator, which under non-stressed conditions is located at the inner membrane (Lee et al., 2008). During acid stress, CadC is cleaved resulting in release of the DNA binding transcriptional regulator (Lee et al., 2008). In this system, intracellular lysine is combined with a proton and converted to cadaverine by CadA. The CadB antiporter exports cadaverine and imports extracellular lysine (Park et al., 1996). Viala et al. (2011) described that the lysine decarboxylase system significantly contributes to *Salmonella* survival following acid shock at pH 2.3 and aerobic growth at pH 4.5. Furthermore, *cadC* is induced during murine infection (Heithoff et al., 1997) and intramacrophage survival (Eriksson et al., 2003) although Viala et al. (2011) reported that the CadA carboxylase is not required for systemic murine infection.

The inducible arginine decarboxylase system is composed of the AdiA arginine decarboxylase, the arginine-agmatine antiporter, AdiC, and the transcriptional regulator, AdiY. In this system, intracellular arginine consumes a proton to form

agmatine, which is exported via AdiC in replacement for extracellular arginine (Kieboom and Abee, 2006, Alvarez-Ordóñez et al., 2010, Álvarez-Ordóñez et al., 2012). Initial studies reported that the system is only induced in anaerobic acidic conditions (Kieboom and Abee, 2006); however, later studies disputed this claim and described the arginine system in aerobic acidic conditions (Alvarez-Ordóñez et al., 2010). In contrast to the lysine decarboxylase, AdiA is dispensable for *Salmonella* growth at pH 4.5 but is highly important for survival at pH 2.3 (Viala et al., 2011).

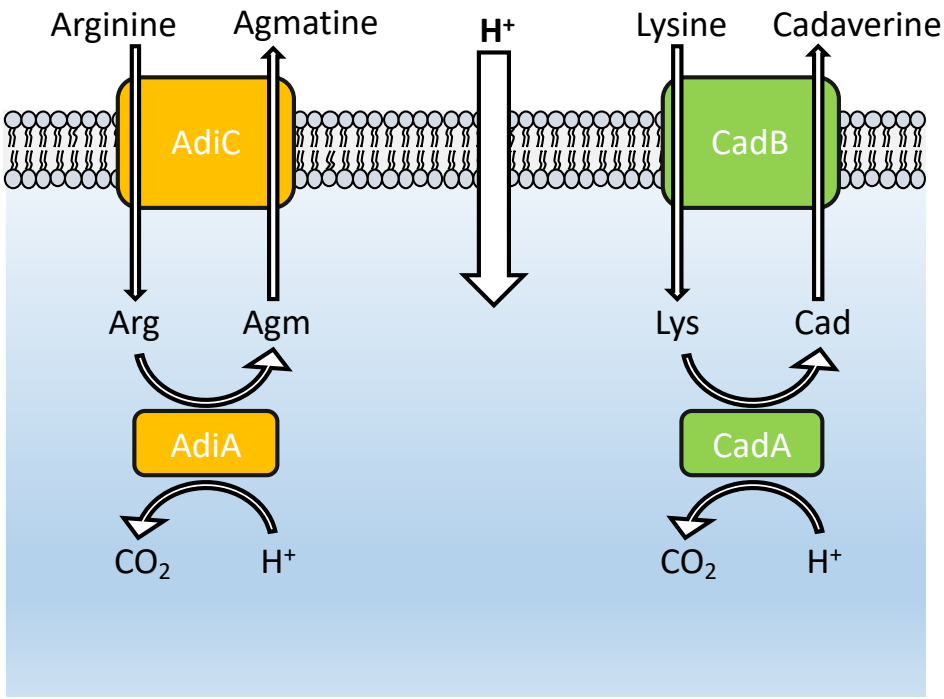


Figure 51. The arginine and lysine decarboxylase systems maintain intracellular pH homeostasis during exposure to acidic environmental conditions. In the presence of acidic conditions, the intracellular pH can increase above optimal levels due to the influx of protons (H^+). In *Salmonella,* the arginine and lysine decarboxylases, AdiA and CadA respectively, combine a proton with their cognate amino acid substrate. The resulting product (agmatine or cadaverine) is exported via an antiporter (AdiC or CadB) in exchange for the import of more arginine or lysine.

Despite the importance of lysine and arginine decarboxylases in acid resistance, *Salmonella* continues to exhibit an ATR in minimal, amino-acid free media, pointing towards a role of additional acid resistance mechanisms (Alvarez-Ordóñez et al., 2010, Ramos-Morales, 2012).

4.1.1.1 Transcriptional regulation of acid shock protein expression

The production of acid shock proteins (ASPs) is crucial for the protection and repair of damaged DNA and proteins in acid shock conditions. Surprisingly, many ASPs are poorly characterised but their general roles include transcriptional and translational regulation, molecular chaperones, cell envelope maintenance and virulence (Álvarez-Ordóñez et al., 2011). While pre-shock adaptation at pH 5.8 induces the homeostatic mechanisms described above, it also induces the expression of 12 proteins including OmpC and OmpF. In contrast, the expression of 52 proteins is significantly changed following transfer of *Salmonella* from pH 7.7 to pH 4.5 (Foster and Hall, 1990, Foster, 1991), including the heat shock chaperonin proteins DnaK and GroEL (Foster, 1991). In the literature, four major bacterial stress responses are reported to be responsible for the induction of ASP gene expression: RpoS, Fur, PhoPQ and OmpR/EnvZ. This section will also address the relationship between acid shock and the extracytoplasmic sigma factor, σ^E.

4.1.1.1.1 RpoS

The RpoS sigma factor was introduced in Chapter 1. It plays a major role in stationary phase survival and contributes to the resistance against multiple stresses, including low pH (Rychlik and Barrow, 2005). Induction of the RpoS stress response at pH 4.3 leads to the expression of at least 40 proteins (Soo Lee et al., 1995). Low pH induction of σ^S occurs by different mechanisms, depending on the acid present (inorganic or organic). Inorganic acid shock induces RpoS-dependent gene expression via inhibition of RpoS proteolysis, by the mouse virulence gene *mviA* (Bearson et al., 1996). In comparison, in the presence of organic acids, σ^S stability and the translation of *rpoS* is increased, which is usually controlled by secondary structures located within the *rpoS* mRNA UTR (Audia and Foster, 2003).

σ^S has been attributed to regulation of ASPs in the exponential and stationary phase ATR. The RpoS regulon is induced during the *Salmonella* exponential phase ATR, when the period of adaptation is at least 60 mins and an *rpoS* deletion renders *Salmonella* highly sensitive to pH 4 (Fang et al., 1992). In stationary phase cells, σ^S appears to increase survival in a pH-independent manner, due to its role in general stationary phase survival (Lee et al., 1994). Coupled to its general stress response role, following adaptation in acidic environments RpoS can also provide *Salmonella* with cross protection to other stresses such as heat, H_2O_2 and osmolarity, although pre-exposure to these same stresses does not provide equal cross protection to acid shock (Soo Lee et al., 1995).

4.1.1.1.2 Fur

Introduced in Chapter 3, the ferric uptake regulator (Fur) was first identified as a transcriptional repressor in the presence of iron, increasing expression of iron transport and metabolism genes only in iron deplete conditions. Later studies demonstrated that Fur also responds to organic acid stress and ∆*fur* mutants are unable to elicit a complete adaptive ATR (Foster, 1991, Foster and Hall, 1992). The link between iron metabolism and the acid response, controlled by Fur, is unclear although low pH and the presence of iron (via Fenton's reaction) can lead to cellular oxidative stress (Rychlik and Barrow, 2005). The iron and acid sensing mechanisms of Fur are understood to be separable; a site directed mutant (H90R) is defective in its iron response but is able to mount a complete ATR (Hall and Foster, 1996). The precise regulon of acid-induced genes regulated by Fur is poorly characterised. Moreover, *fur* mutants are not significantly attenuated during murine infection, highlighting the overlapping nature of the other *Salmonella* ATR mechanisms (Garcia-del Portillo et al., 1993).

4.1.1.1.3 PhoPQ

Expression of PhoP is induced by pH 4.4 (Bearson et al., 1998) and PhoQ functions as a direct pH sensor in *Salmonella* (Prost et al., 2007). Investigations into the mechanism of PhoPQ induction revealed interesting differences between activation by cationic AMPs and acidic pH. Similarly to Fur, site-directed mutagenesis (SDM) has revealed that disruption and loss of cationic AMP activation by PhoQ does not lead to loss of acidic pH activation (Prost et al., 2007). In fact, Prost et al. (2007) reported that the two mechanisms of activation are distinct and can be additive.

Unadapted *Salmonella* ∆*phoP* mutants are highly sensitive to pH 3.3 and show a reduced ability to mount an adaptive ATR compared to WT (Foster and Hall, 1990). Moreover, a later study reported that PhoP is important for inorganic acid resistance but is not required for survival following organic acid shock (Bearson et al., 1998). PhoPQ is highly important for intramacrophage survival and acidification of the SCV also contributes to the ability of *Salmonella* to proliferate in this environment. It is suggested that acidification is an important environmental factor for induction of the PhoPQ regulon and therefore for *Salmonella* pathogenesis (Audia and Foster, 2003).

4.1.1.1.4 OmpR/EnvZ

EnvZ and OmpR form the sensor kinase and response regulator components of the OmpR/EnvZ TCS. EnvZ is best characterised for its ability to sense low osmolarity, leading to activation of OmpR and increased expression of its regulon of genes which

include outer membrane porins. In addition to its osmolarity related functions, OmpR has been previously described as an ASP (Bang et al., 2000). OmpR is required for the stationary phase ATR (Rychlik and Barrow, 2005); Δ*ompR* mutants exhibit an exponential phase adaptive ATR but are unable to survive in stationary phase (Bang et al., 2000). Although originally described as a TCS, EnvZ is not required for OmpR induction of *ompC* and *ompF* in low pH conditions, suggesting that additional activators of *ompR* exist in this environment (Rychlik and Barrow, 2005). When residing in the SCV, *Salmonella* exploits the acidic environment for pathogenesis and *Salmonella* has been shown to maintain acidity of the cytoplasm in an OmpR-dependent manner; OmpR represses the *cadCBA* genes, preventing homeostatic control of intracellular pH. Although this appears counter-intuitive, *Salmonella* uses cytoplasmic acidification as an environmental cue for the expression of the SPI-2 T3SS secretion system (Kenney, 2019).

4.1.1.1.5 RpoE and the ATR

RpoE is not associated to be one of the 'core' regulators of ASPs. However, Muller et al. (2009) demonstrated that σ^E was critically required for survival during acid stress and its expression is increased under these conditions. During acid stress, σ^E induction occurs via a non-canonical mechanism first indicated in a study that identified that *degS* mutants are not as attenuated as an *rpoE* mutant in mice (Rowley et al., 2005), suggesting that *rpoE* activation can still occur. A later study confirmed that during acid stress, induction of the σ^E regulon still occurs in *degS* mutants (Muller et al., 2009). This novel mode of σ^E activation does still require the RseP site II protease (Muller et al., 2009). This mechanism has only been observed in *Salmonella* during acid stress and interestingly, there are currently no reports that have investigated whether this is also the case in *E. coli*, despite this bacterium being the focus of the majority of σ^E characterisation studies to date.

4.1.2 *Salmonella* and reactive oxygen species

In contrast to acid stress, RpoE and its regulon of genes are well characterised with respect to their roles in resistance to oxidative stress. The intramacrophage environment is extremely hostile and in addition to acidification, phagocytes are producers of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) and reactive nitrogen species (RNS) such as nitric oxide (NO). Superoxide is produced by NADPH oxidase, via the reduction of molecular oxygen, which is activated following phagocytosis and PRR signalling (Nguyen et al., 2017) while NO is produced by the inducible nitric oxide synthase enzyme (iNOS).

The NADPH oxidase (Phox) is formed of five subunits (p22, p40, p47, p67 and gp91) and its generation of superoxide plays a central role in clearing infections (Belambri et al., 2018). This is highlighted in patients with chronic granulomatous disease (CGD), who suffer from recurrent and persistent bacterial and fungal infections (Holland, 2010). Mutations (missense, nonsense, frameshift, splice or deletion) in any of the five genes that encode NADPH oxidase subunits are responsible for CGD and *Salmonella* is the second most common cause of bacterial infection in CGD patients (Vazquez-Torres and Fang, 2001b). Superoxide is an important precursor to the formation of other ROS. For example, superoxide dismutation leads to the production of hydrogen peroxide (H_2O_2), while O_2^- can also react with NO to form peroxynitrite ($ONOO^-$). In its anionic form, at neutral pH, superoxide cannot cross bacterial membranes, but the acidic intracellular environment of phagocytes leads to protonation of superoxide and allows for its entry into the bacterial cytoplasm (Imlay, 2019). In contrast, H_2O_2 diffuses across bacterial membranes and OM porins such as OmpD have also been shown to facilitate its entry into the cell (Morales et al., 2012). In addition, H_2O_2 can react with iron and copper in the Fenton reaction to generate superoxide (Storz et al., 1990, Buchmeier et al., 1995).

Resistance to oxidative stress is essential for *Salmonella* survival within phagocytes. The primary killing mechanism of ROS is DNA damage but studies have suggested that ROS may also act on extracellular targets, although the precise targets are still unclear (Craig and Slauch, 2009, Fang, 2011, Slauch, 2011). The major roles of bacterial oxidative defences are to either eliminate the presence of ROS or to repair the damage caused (Hébrard et al., 2009). To achieve this, catalase activity (primarily regulated by OxyR), DNA repair mechanisms and stress responsive chaperones and proteases (regulated by OxyR, SoxR, σ^E and σ^S) are essential.

The H_2O_2 detoxification system in *Salmonella* involves three functionally redundant catalases (KatE, KatG and KatN) and two alkyl hydroperoxide reductases (AhpC and TsaA) (Hébrard et al., 2009). Deletion of these five key components generated a strain, designated HpxF-, that is extremely sensitive to H_2O_2 and is attenuated in macrophages (Hébrard et al., 2009). Superoxide detoxification involves the superoxide dismutase (Sod) *sodCI* and *sodCII* and they are required for the conversion of superoxide to H_2O_2 and water. Although this results in endogenous production of H_2O_2, the H_2O_2 detoxification systems described above are capable of managing this by-product (Imlay, 2019).

4.1.3 Aims

Hydrogen peroxide (H_2O_2) and acid shock induce the expression of a subset of genes in *Salmonella*, that include heat shock proteins such as DnaK and GroEL (Morgan et al., 1986, Foster, 1991). Since σ^E and its regulon of genes are important for *Salmonella* resistance to oxidative stress (Testerman et al., 2002) and highly expressed during acid shock (Muller et al., 2009) we hypothesised that the σ^E-regulated genes in this study could contribute to *Salmonella* resistance to acid and oxidative stress.

The *Salmonella* specific nature of *agsA* and *STM1250* suggests that they may provide a *Salmonella*-specific advantage within the niches that it occupies within a host, for example within the SCV. As for the previous chapter, it was hypothesised that IbpAB functionally overlap with the *Salmonella* specific proteins and may also provide protection to host-associated stresses.

Therefore, this chapter aimed to extend the physiological characterisation of the *ibpA*, *ibpB*, *STM1250* and *agsA* genes presented in Chapter 3 into an investigation of their contributions to *Salmonella* pathogenesis.

These aims were achieved though:

- Assessing the ability of the different *Salmonella* mutants to mount an ATR in the presence of organic and inorganic acids, in exponential and stationary phase cells.
- Investigating the sensitivity of the mutant strains to different sources of oxidative stress (hydrogen peroxide, paraquat, tellurite, indole).
- Macrophage infection assays to determine the contribution of the sHsps and STM1250 to *Salmonella* intracellular survival.
- Investigation of proposed PhoPQ regulation of *STM1250* by qRT-PCR.

4.2 Results

4.2.1 *STM1250* and *agsA* are located downstream of the SPI-11 genomic region

The *Salmonella* specific STM1250 and AgsA are conserved differently among *Salmonella* serovars (3.2.3). In this chapter, the genomic location of these genes was assessed to determine whether their location and neighbouring genes could enable a better elucidation of their cellular roles in *Salmonella*.

Prior to the publication of the *Salmonella* Typhimurium LT2 genome, *Salmonella* pathogenicity islands 1-5 had been previously described and studied to different extents (Shea et al., 1996, Wood et al., 1998, Wong et al., 1998, Hensel et al., 1998, Blanc-Potard et al., 1999). However, publication of the genome led to the identification of numerous other genomic islands and in particular new SPIs (McClelland et al., 2001). Of the pathogenicity islands McClelland et al. (2001) described that fifteen are located next to a transfer RNA, indicating that they were acquired during *Salmonella* evolution by horizontal gene transfer. One such example of a SPI located adjacent to a tRNA (tRNA-Arg) is SPI-11, predicted to start at STM1239 and to date, described as containing seven genes (*STM1239*, *envF*, *msgA*, *envE*, *STM1243*, *pagD*, and *pagC*) (McClelland et al., 2001). McClelland et al. (2001) presents the *STM1250* and *agsA* genes in context of SPI-11 and the surrounding genomic location, coloured based on percentage GC content. In addition to proximity to a tRNA, percentage GC content is an indicator of a SPI. Pathogenicity islands have a lower percentage GC content in comparison to the genome (Marcus et al., 2000, Hayek, 2013), in the case of SPI-11 the average percentage GC content is 45.9% compared to the *Salmonella* genome average of 52%.

In Figure 52 the genes shown in green contain a lower percentage GC than the *Salmonella* genome, suggesting that they have been acquired during *Salmonella* evolution and that they may form part of a pathogenicity island. It can be observed that six genes directly downstream of the tRNA-Arg are classified as low GC: *pliC*, *STM1250*, *agsA*, *STM1252*, *STM1253* and *STM1254*, suggesting that in fact, SPI-11 extends beyond the currently described region.

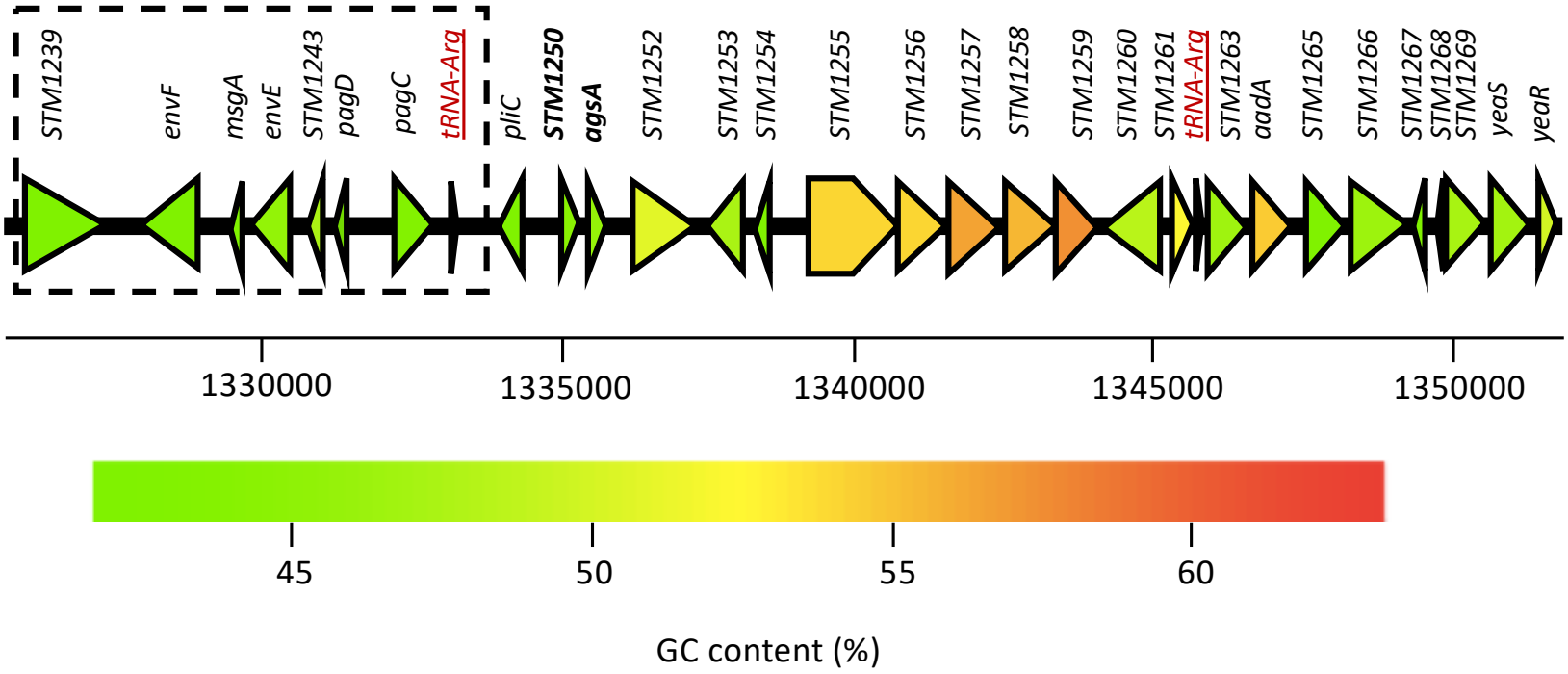


Figure 52. The genomic location of *STM1250* and *agsA* within the context of SPI-11. *STM1250* and *agsA* are located downstream of the currently described SPI-11 region (indicate by a broken square). Two tRNA-Arg which suggest a region of horizontal gene transfer are underlined. Genes are coloured based on their percentage GC content, as shown in the associated legend. Figure adapted from XBase (xbase.warwick.ac.uk).

4.2.2 Deletion of *ibpA*, *ibpB*, *STM1250* or *agsA* does not significantly reduce overall growth of *Salmonella* in ATR assay minimal media

Prior to performing acid shock assays, growth curves were performed with WT and mutant strains in order to assess their ability to grow in the assay media (no-carbon essential (NCE) minimal media supplemented with 0.4% (w/v) glucose) at pH 7.0.

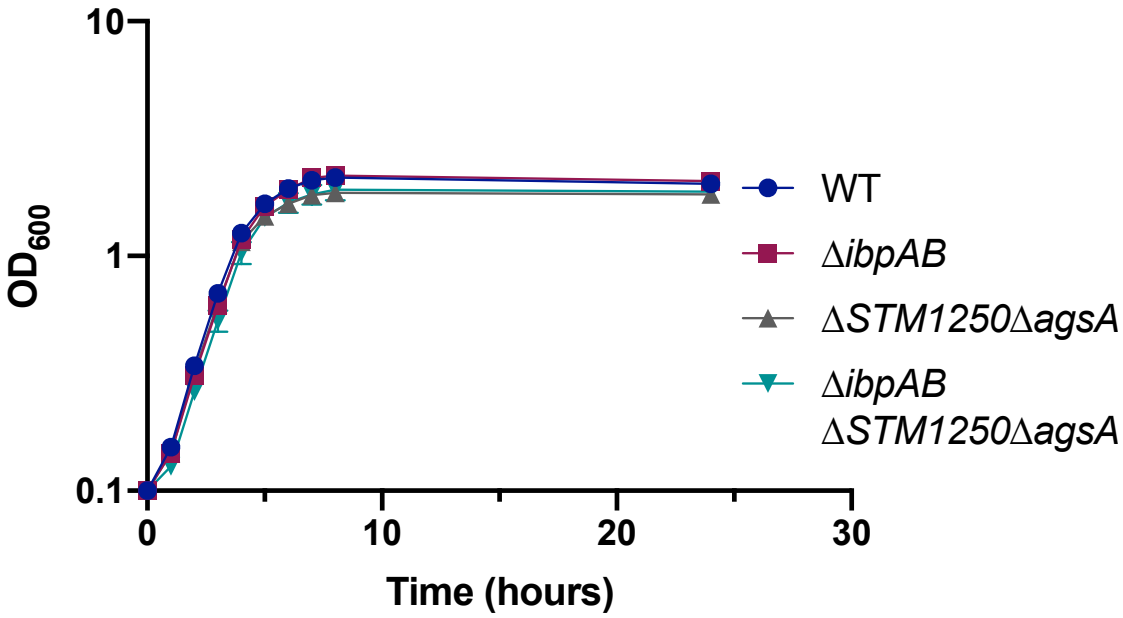


Figure 53. Overall growth of WT and *Salmonella* mutants is not significantly reduced in NCE medium, pH 7.0. NCE medium was inoculated with bacteria and incubated at 37 °C for 24 hours. Samples (1 mL) were taken hourly for the first 8 hours and OD_{600} readings were measured. A final 1 mL sample and OD_{600} was also read after 24 hours of incubation. Data points are representative of the means of three separate experiments performed in duplicate. Error bars are SEM.

Growth of both the WT and the mutant strains were equivalent in NCE pH 7.0 (Figure 53). This confirmed that any subsequent differences between WT and mutants during acid shock assays could be attributed to the acidity of the growth environment and not due to an inability of the strains to grow in the assay minimal media.

4.2.3 The adaptive ATR of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is reduced compared to WT

As an initial screen, the Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant was assessed for its ability to survive acidic challenge at pH 3.0 either with (to induce the ATR) or without a pre-incubation at pH 4.4. Bacteria were grown overnight in NCE medium (pH 7.0) before being transferred to acid shock or adaption media. For acid shock cultures, the bacteria were incubated in NCE pH 3.0 and samples were taken at 30, 60 and 90 mins. For adapted ATR cultures, the bacteria were incubated in NCE pH 4.4 for 2 hours, and were then transferred to NCE pH 3.0 for up to 90 mins with regular samples taken as above.

Unadapted bacteria displayed a reduction in percentage survival of approximately 30% over the course of the 90 min incubation. Overall, the WT and quadruple mutant behaved equally and there was no difference in the ability of the mutant to survive the acid challenge in comparison to WT (Figure 54). In contrast, although overall reduction in percentage survival of the acid adapted bacteria after 90 mins was similar for both strains, a significant difference in percentage survival between the WT and mutant was observed after 30 mins incubation at pH 3.0. Whilst the quadruple mutant displayed a steady decline in percentage survival over the course of the experiment, the survival of the WT in fact increased between 0 and 30 mins, before a reduction for the remainder of the experiment (Figure 54).

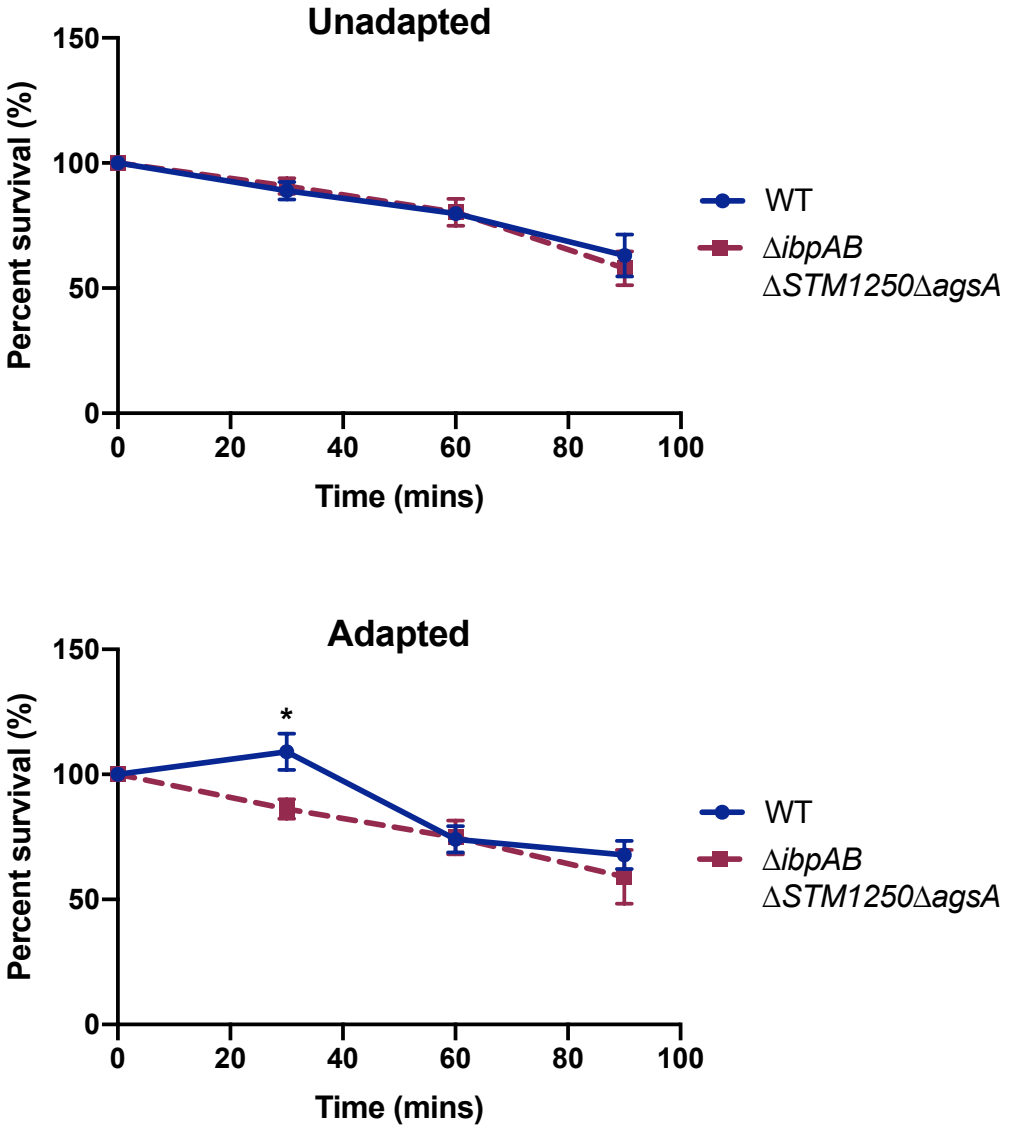


Figure 54. Survival of WT *S.* Typhimurium after 30 mins acid shock at pH 3.0 is significantly increased following adaptation at pH 4.4, compared to the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant. Unadapted cultures were transferred directly from pH 7.0 to acid shock media (adjusted to pH 3.0 with HCl) and incubated for 60 mins. Adapted cultures were transferred from pH 7.0 to adaption media (adjusted to pH 4.4 with HCl) for 2 hours, before being transferred to acid shock media (adjusted to pH 3.0 with HCl) for 60 mins. Samples (1 mL) were taken at 30 mins, 60 mins and 90 mins and percentage survival was calculated relative to a t=0 control sample. Data points are the means of three separate experiments performed in duplicate and error bars are SEM. Data at each time point analysed by Student's *t*-test, * $p < 0.05$.

4.2.4 STM1250 is important for the *Salmonella* stationary phase adaptive ATR

The significant increase in the survival of the adapted WT compared to the adapted Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was intriguing and warranted further validation by additional time point sampling. Therefore, following a 2 hour adaptation period at pH 4.4, additional samples were taken at 15, 30, 45 and 60 mins after acid shock at pH 3.0. The initial results (Figure 54) were confirmed; the percentage survival of the WT was observed to increase during the first 30 mins of acid shock while the quadruple mutant displayed a steady decline (Figure 55A).

In order to determine their individual importance and address any potential functional overlap between the pairs of genes, the double (Δ*ibpAB* and Δ*STM1250*Δ*agsA*) mutants were also tested. Interestingly, this indicated that IbpA and IbpB are not involved in the *Salmonella* ATR in the defined conditions of this study, but STM1250 and/or AgsA are critical for the ability of acid-adapted *S.* Typhimurium to replicate in pH 3.0 acidic conditions (Figure 55C).

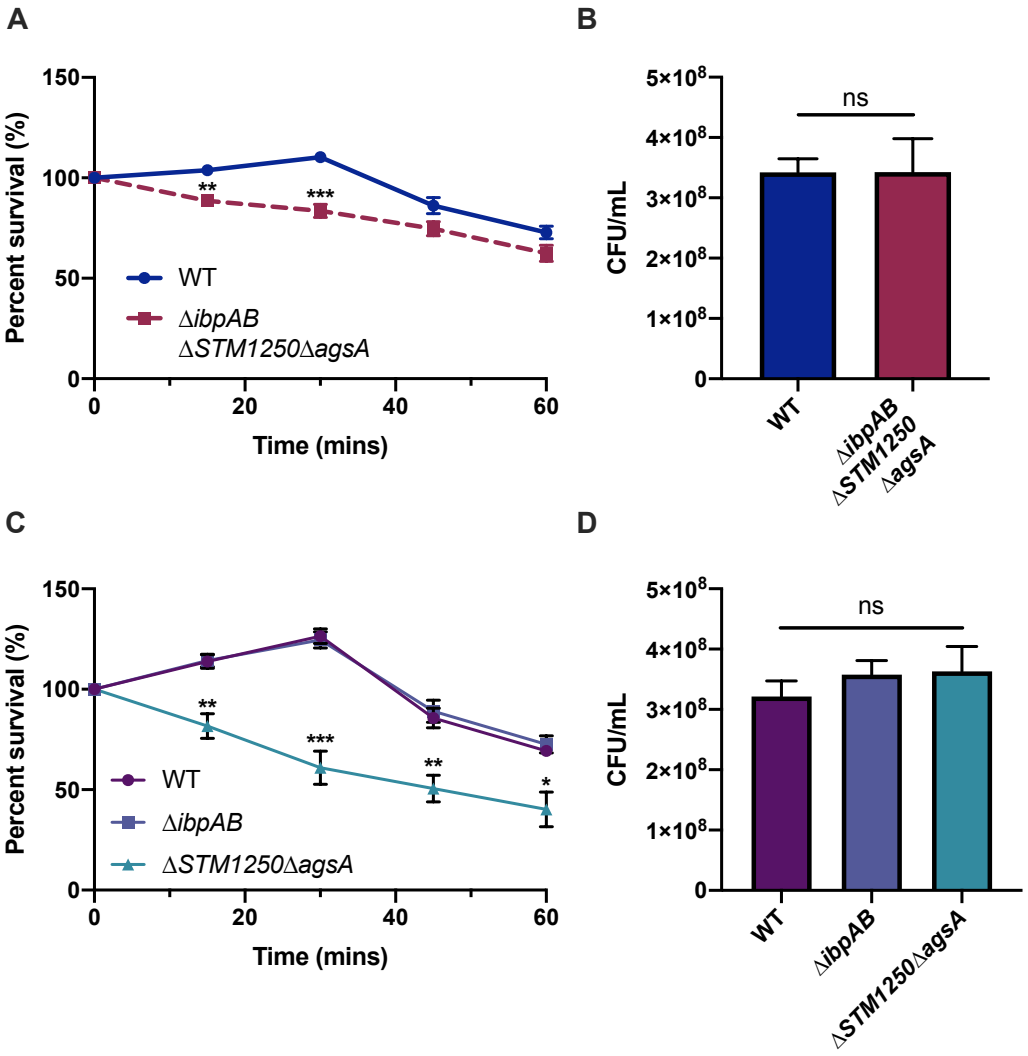


Figure 55. Survival of the SL1344 Δ*ibpAB*Δ*STM1250*Δ*agsA* and Δ*STM1250*Δ*agsA* mutants are significantly reduced compared to WT after 30 mins and 60 mins acid shock, respectively. All bacterial cultures were adapted at pH 4.4 for 2 hours before exposure to acid shock media (adjusted to pH 3.0 with HCl) for up to 60 mins. Samples were taken every 15 mins and percentage survival compared to 0 hour controls (A and C). CFU/mL for each 0 hour control confirmed that adaption for 2 hours does not have a negative effect on the survival of the strains tested (B and D). Data points are the means from three separate experiments performed in duplicate, error bars are SEM. Statistical analysis between two groups (WT vs Δ*ibpAB*Δ*STM1250*Δ*agsA*) were performed by Student's *t*-test and statistical analysis between multiple groups (WT vs Δ*ibpAB* vs *STM1250*Δ*agsA*) were performed by one-way ANOVA with Tukey's post-test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

As a result, the Δ*STM1250* and Δ*agsA* single mutants were further assessed for their ability to survive pH 3.0 acid shock (Figure 56). Interestingly, the *agsA* mutant behaved similarly to the WT. However, the percentage survival of the *STM1250* deletion mutant was similar to the Δ*STM1250*Δ*agsA* mutant. These findings indicated a novel role for STM1250 in the ability of *Salmonella* to mount an ATR at pH 3.0.

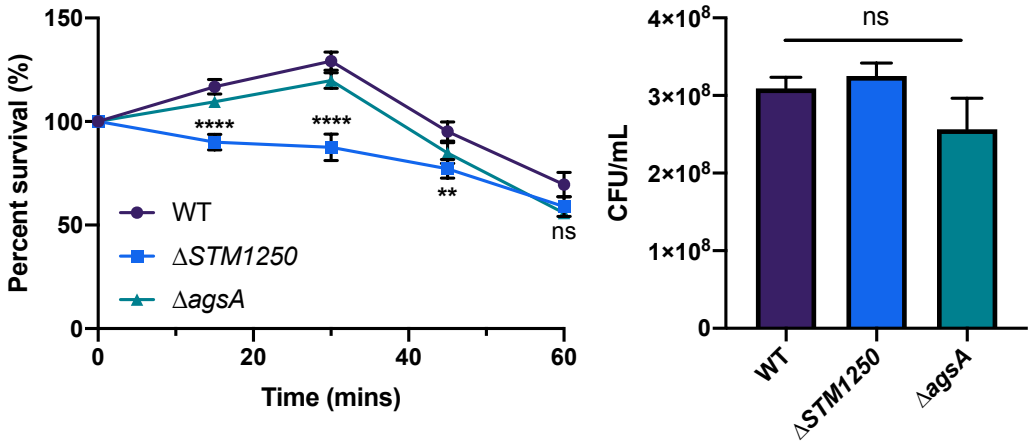


Figure 56. The *S.* Typhimurium Δ*STM1250* mutant is significantly reduced in its ability to survive acid shock conditions (pH 3.0) following adaption in pH 4.4 media, compared to WT and Δ*agsA*. All bacterial cultures were adapted at pH 4.4 for 2 hours before exposure to acid shock media (adjusted to pH 3.0 with HCl) for up to 60 mins. Samples were taken every 15 mins and percentage survival compared to 0 hour controls. The CFU/mL for each 0 hour control confirmed that adaption for 2 hours does not have a negative effect on the survival of the strains tested. Data points are the means from three separate experiments performed in duplicate, error bars are SEM. Statistical analysis was performed by one-way ANOVA with Tukey's post-test, ns $p > 0.05$, ** $p < 0.005$, **** $p < 0.0001$ Δ*STM1250* vs WT.

4.2.4.1 Overexpression of STM1250 fully complements the acid sensitivity of the Δ*STM1250* mutant

Phenotypic complementation was achieved by IPTG-inducible overexpression of *STM1250*. The WT and Δ*STM1250* strain carrying the empty vector (pET-Duet1) behaved in the same manner as previously, with a significant reduction in survival of the mutant compared to WT. In comparison, overexpression of STM1250 increased survival of the WT and Δ*STM1250* mutant compared to WT + empty vector levels;

survival of both overexpression strains was significantly greater than WT after 45 mins and 60 mins incubation at pH 3.0 (Figure 57).

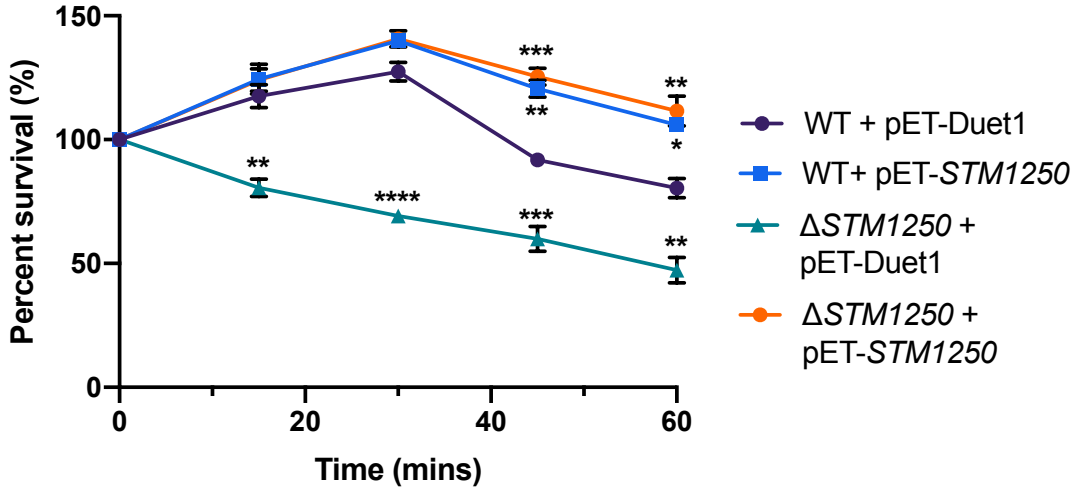


Figure 57. Overexpression of STM1250 recovers acid sensitivity of the Δ*STM1250* mutant and significantly increases survival above WT levels. All bacterial cultures were adapted at pH 4.4, in the presence of 1 mM IPTG, for 2 hours before exposure to acid shock media (adjusted to pH 3.0 with HCl), in the presence of 1 mM IPTG, for up to 60 mins. Data points are the means of three separate experiments performed in duplicate and error bars are SEM. Statistical analysis was performed by one-way ANOVA with Tukey's post-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ vs WT + pET-Duet-1.

4.2.5 STM1250 is required for the exponential phase acid tolerance response in *S.* Typhimurium

Initial ATR studies were performed using stationary phase cells. As described in the introduction to this chapter, the precise mechanisms of the *Salmonella* ATR are different depending on the growth phase, with different regulators responsible in each phase. To this end, the role of STM1250 was also investigated using exponential phase cultures.

Bacteria were grown to mid-exponential phase (OD_{600} 0.4) in NCE, pH 7.0 and as previously, were transferred to adaptation media, adjusted to pH 4.4 with HCl for 2 hours. Following adaptation, bacteria were incubated in NCE, pH 3.0 for 60 mins. In the same manner as stationary phase cultures, there was a significant difference

between percent survival of the Δ*STM1250* mutant compared to WT at all time points sampled (Figure 58).

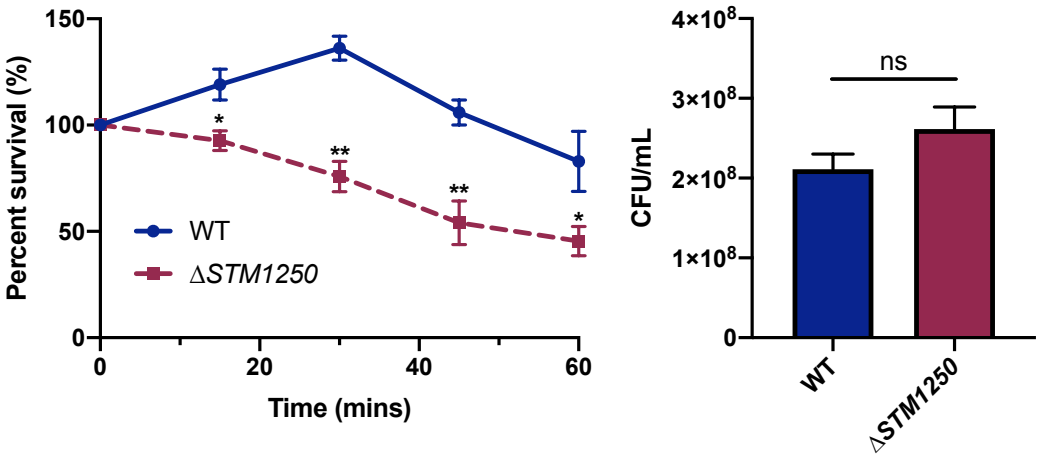


Figure 58. The survival of the acid-adapted *S.* Typhimurium Δ*STM1250* mutant is significantly reduced compared to WT when exposed to acid shock conditions (pH 3.0) during exponential growth. Exponential phase cultures were adapted at pH 4.4 for 2 hours before exposure to acid shock media (adjusted to pH 3.0 with HCl) for up to 60 mins. Samples were taken every 15 mins and percentage survival compared to 0 hour controls. CFU/mL for each 0 hour control confirmed that the adaption period does not affect the survival of the WT or mutant. Data points are the means from three separate experiments performed in duplicate, error bars are SEM. Statistical analysis was performed by Student's *t*-test, ns $p > 0.05$ * $p < 0.05$, ** $p < 0.005$.

4.2.6 STM1250 is involved in the stationary phase ATR in the presence of acetic acid

Since all prior ATR assays utilised HCl to adjust the pH of the adaptation and shock media, the effect of an organic acid, acetic acid, on the survival of WT and Δ*STM1250* mutant was also assessed. When bacteria were adapted at pH 4.4 (adjusted with acetic acid), there were no viable cells remaining after 2 hours. Therefore, for acetic acid assays, adaptation media was adjusted to pH 6.0 and acid shock media was adjusted to pH 5.0. Despite the different acid used and final pH of the media, survival of the WT and Δ*STM1250* mutant was replicated; the percentage survival of the mutant was significantly reduced compared to WT after 15 mins and 30 mins incubation at pH 5.0. However, analysis of the CFU/mL following the 2 hour

adaptation period also revealed a significant reduction in CFU/mL of the Δ*STM1250* mutant compared to WT (Figure 59).

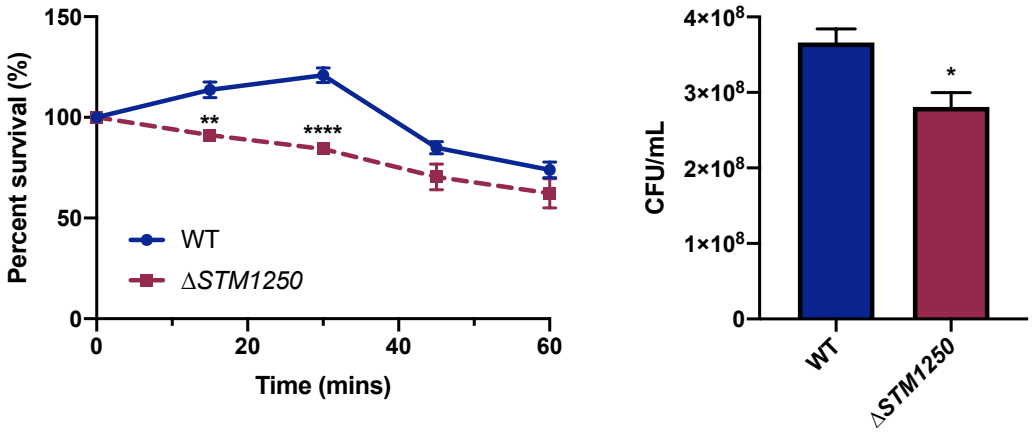


Figure 59. STM1250 contributes to the *S.* Typhimurium ATR in the presence of acetic acid. All bacterial cultures were adapted at pH 6.0 for 2 hours before exposure to acid shock media (adjusted to pH 5.0 with acetic acid) for up to 60 mins. Samples were taken every 15 mins and percentage survival compared to 0 hour controls. CFU/mL calculated for each 0 hour sampled (pre-acid shock) identified that adaption for 2 hours reduced overall survival of the Δ*STM1250* mutant compared to WT. Data points are the means from three separate experiments performed in duplicate, error bars are SEM. Statistical analysis was performed by Student's *t*-test, * $p < 0.05$, ** $p < 0.005$ and **** $p < 0.0001$.

4.2.7 Determining the contribution of RpoE and PhoP to stationary phase *Salmonella* acidic minimal media

At present, the only experimentally confirmed regulator of *STM1250* is RpoE (Skovierova et al., 2006), although as indicated in the previous chapter, regulation by PhoPQ is also predicted (Monsieurs et al., 2005). In order to investigate the potential transcriptional regulators controlling *STM1250* expression during acid stress, the responses of the Δ*phoP* and Δ*rpoE* regulator mutants were also assessed in the *Salmonella* stationary phase ATR, in media acidified with HCl.

4.2.7.1 Construction of a *Salmonella* Δ*phoP* mutant

A *phoP* deletion mutant was made in the SL1344 background by lambda red recombination followed by P22 transduction. The *phoP* gene was replaced with a kanamycin resistance cassette amplified from the plasmid pKD4.

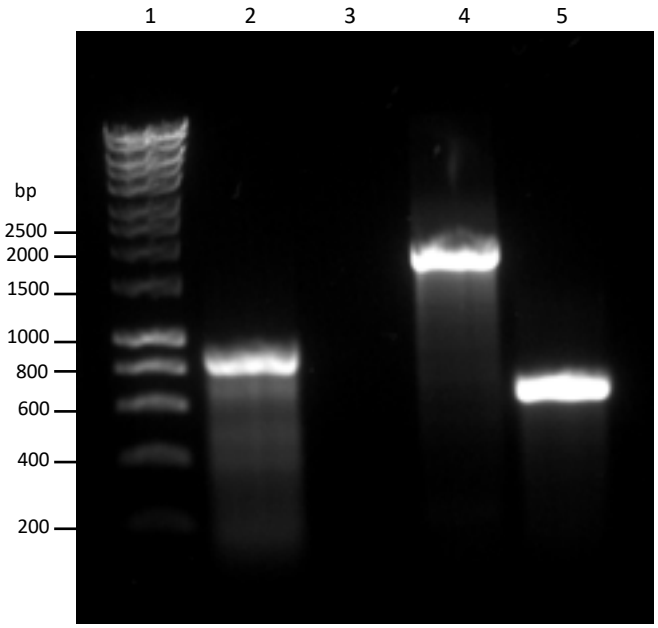


Figure 60. Verification of the construction of an SL1344 Δ*phoP* mutant following lambda red mutagenesis and P22 transduction. Lane 1: 1 kb ladder (Bioline). Lane 2: WT *phoP* (expected size 787 bp). Lane 3: WT with internal kanamycin cassette primer (no band expected). Lane 4: *phoP* mutant (expected size 1977 bp). Lane 5: *phoP* mutant with internal kanamycin cassette primer (expected size 599 bp)**.**

4.2.7.2 RpoE, but not PhoP, is critical for stationary phase *Salmonella* survival in minimal media acidified with HCl

Interestingly, Figure 61 shows that despite being previously linked to an inorganic acid associated ATR, the Δ*phoP* mutant behaved in the same manner as the WT. In contrast, percentage survival of the Δ*rpoE* mutant was significantly reduced at all time points sampled during acid shock at pH 3.0. Despite the fact that it is not readily described in the literature as one of the core ATR regulators, the Δ*rpoE* mutant was no longer viable after 60 mins exposure to pH 3.0. Analysis of the CFU/mL after adaptation of cultures at pH 4.4, but prior to acid shock, also revealed a significant reduction in survival of the Δ*rpoE* mutant compared to WT. Put together, these findings suggested that expression of *STM1250* in ATR-inducing conditions is controlled by σ^E and not the PhoPQ TCS.

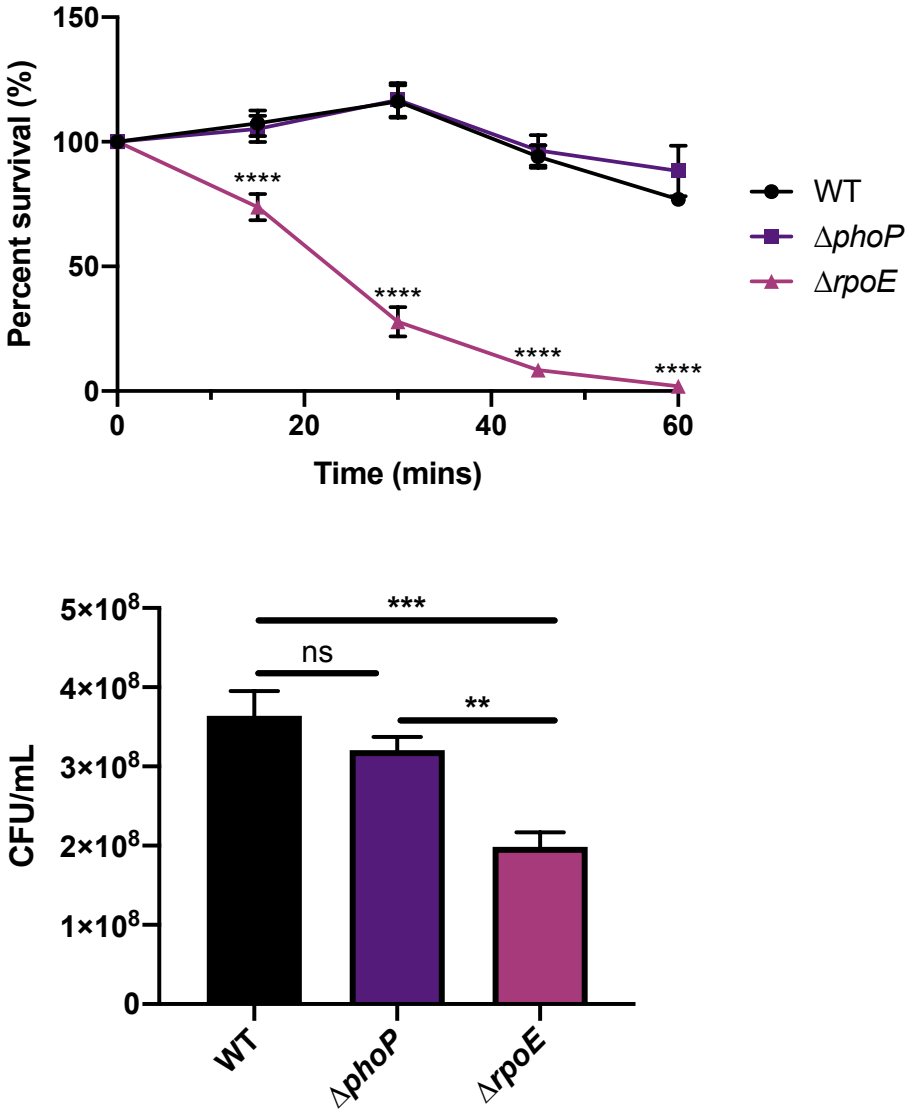


Figure 61. *Salmonella* ∆*rpoE* mutant is highly sensitive to acid shock (pH 3.0) and acid adaption (pH 4.4) conditions but PhoP is not required for survival. All bacterial cultures were adapted at pH 4.4 (adjusted with HCl) for 2 hours before exposure to acid shock media (adjusted to pH 3.0 with HCl) for up to 60 mins. Samples were taken every 15 mins and percentage survival compared to 0 hour controls. CFU/mL for each 0 hour control confirmed that adaption for 2 hours does not have a negative effect on the survival ∆*phoP* but there was a significant difference in CFU/mL of the ∆*rpoE* mutant compared to WT. Data points are the means from three separate experiments performed in duplicate, error bars are SEM. Statistical analysis was performed by one-way ANOVA with Tukey's post-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$ ∆*STM1250* vs WT.

4.2.8 The quadruple mutant is significantly more sensitive to H_2O_2 compared to WT in aerobic and anaerobic conditions

As described in the introduction, the overall contributions of σ^E and its regulon to the ATR have received limited attention in comparison to roles in oxidative stress resistance.

In terms of the specific proteins of interest in this thesis, in *E. coli* both IbpA and IbpB have been shown to be involved in resistance to reactive oxygen species (ROS) and their expression is strongly induced by treatment with H_2O_2 (Zheng et al., 2001). However, the specific roles of IbpA and IbpB, nor STM1250 or AgsA, have been investigated in *Salmonella* resistance to oxidative stress.

4.2.8.1 Sensitivity of *Salmonella* to H_2O_2 in aerobic conditions

In the literature, a wide range of concentrations are used when assessing bacterial sensitivity to H_2O_2. Such concentrations can depend on the bacterium of interest and the sensitivity method being conducted. For example, H_2O_2 shock assays in which H_2O_2 is added to exponentially growing cultures typically use H_2O_2 in the µM – mM concentration range, while growth curves are performed in the mM concentration range (Morales et al., 2012).

In this study, to determine an appropriate H_2O_2 concentration for growth curve assays, and to determine the effect of an increasing H_2O_2 concentration on the growth of WT *Salmonella,* growth curves were performed in LB supplemented with H_2O_2 at a final concentration of 3 mM, 6 mM or 9 mM. As an initial screen, the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was also grown in the same increasing concentrations of H_2O_2. The growth curve results (Figure 62) indicated that WT *Salmonella* is minimally sensitive to 3 mM and 6 mM H_2O_2 but is increasingly more sensitive to the high concentration of 9 mM H_2O_2. In contrast, the mutant appeared to be highly sensitive to 6 mM and 9 mM H_2O_2 and growth was only mildly limited by 3 mM H_2O_2. Based on these initial findings, all further growth curve assays were performed using a final concentration of 6 mM H_2O_2.

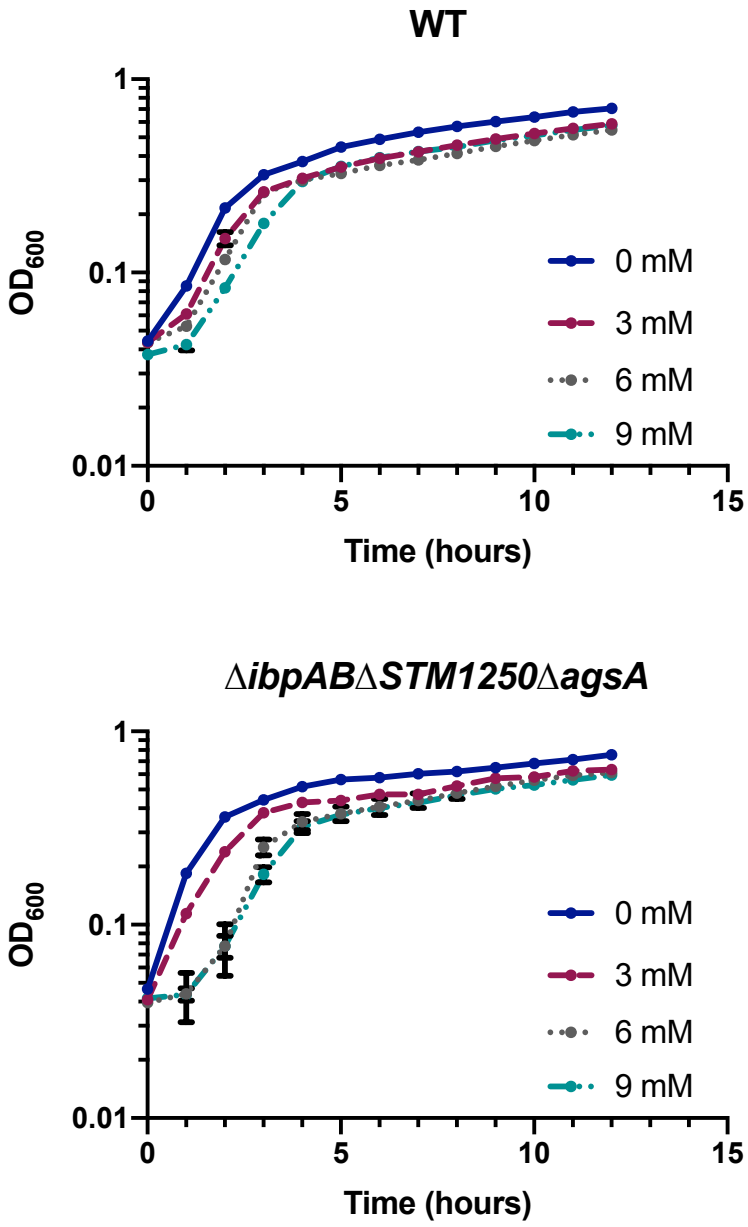


Figure 62. Concentration test for determination of H_2O_2 concentration to be used in growth sensitivity assays. WT *Salmonella* growth is not substantially affected by the addition of 3 mM or 6 mM H_2O_2, but is reduced by 9 mM H_2O_2 in comparison to growth in LB only (0 mM). Growth of the Δ*ibpAB*Δ*STM1250*Δ*agsA* is slightly reduced by 3 mM H_2O_2 and substantially reduced by 6 mM and 9 mM H_2O_2 during the first 2 hours of incubation. Bacteria were grown aerobically in LB in a 24 well plate at 37 °C for 12 hours. Data points are the means of three separate experiments and error bars are SEM (where error bars are not visible they are smaller than the symbols).

In order to investigate the contribution of the four stress responsive proteins to the growth phenotype of the quadruple mutant, growth curves for the single mutants (Δ*ibpA*, Δ*ibpB*, Δ*STM1250* and Δ*agsA*) were conducted in LB containing either 0 mM or 6 mM H_2O_2.

Growth curves (Figure 63) indicated that growth of the single mutants was slightly compromised during the first 4 hours with the greatest difference in growth during this period observed for the Δ*ibpA* mutant. The initial growth rate was calculated for each condition. Although the growth rates of the Δ*ibpA* and Δ*ibpB* mutants were reduced compared to WT, statistical analysis (one-way ANOVA) determined that these differences were not significant (Figure 64).

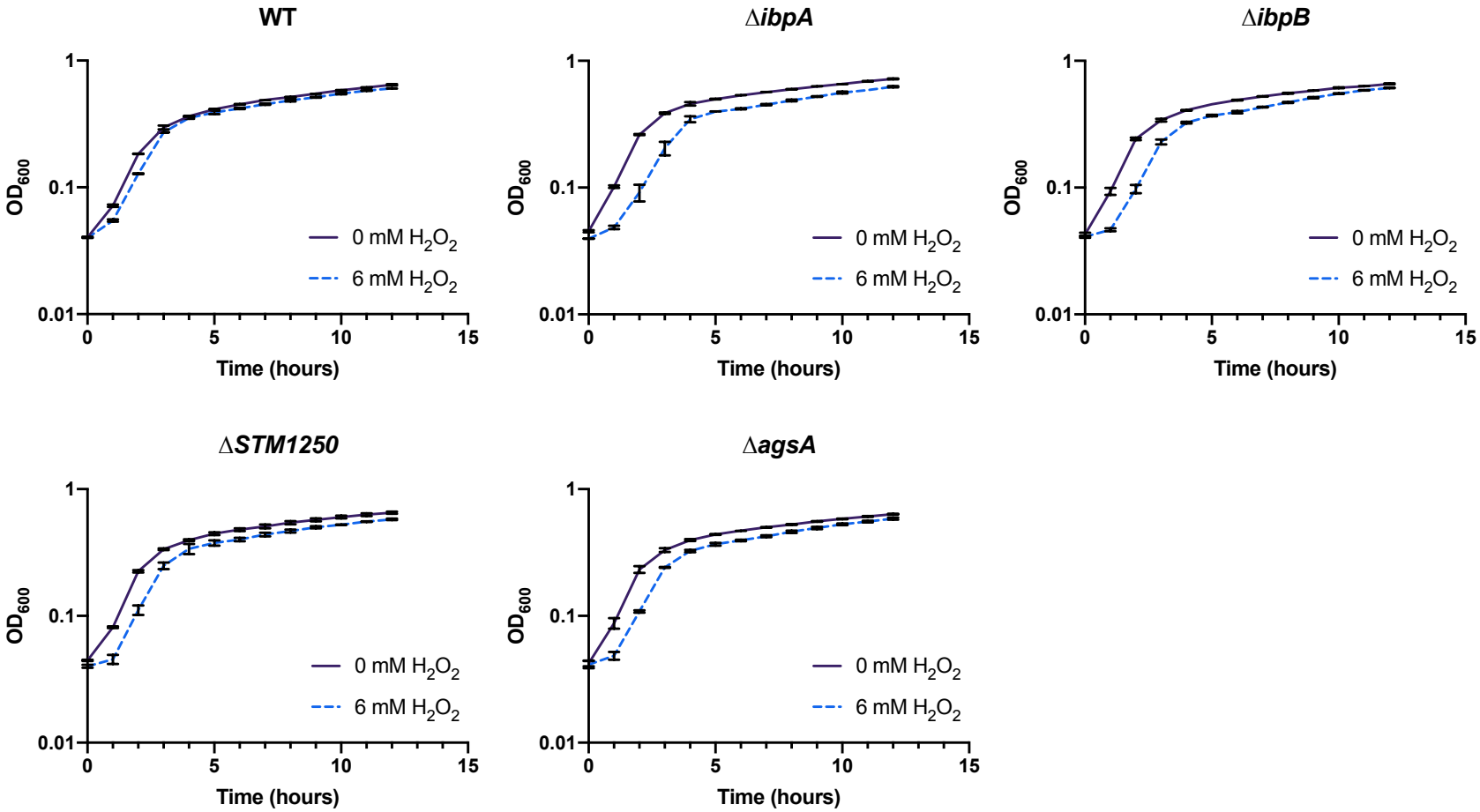


Figure 63. Single deletion mutants *ibpA*, *ibpB*, *STM1250* and *agsA* are not sensitive to 6 mM H_2O_2. Bacteria were grown in 24-well plates in LB containing 0 mM or 6 mM H_2O_2 in LB and incubated at 37 °C in a plate reader for 12 hours. OD_{600} readings were taken hourly. Data is representative of three separate experiments performed in duplicate and error bars are SEM.

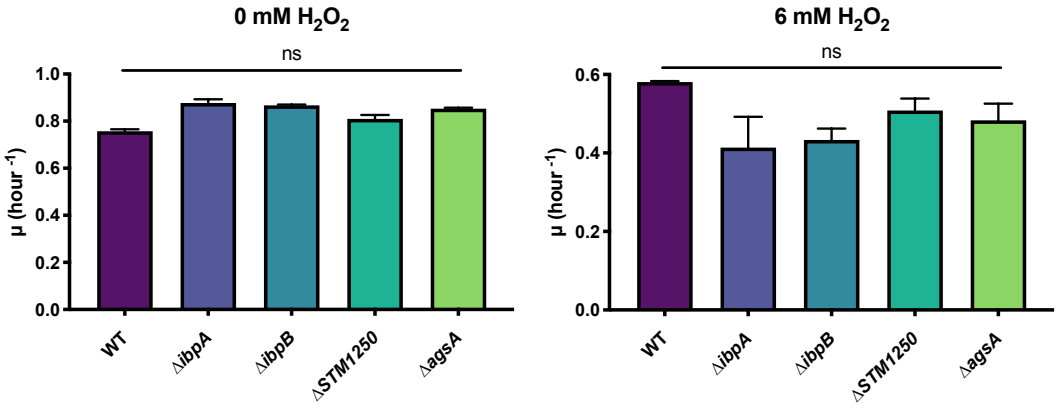


Figure 64. The initial growth rates of the Δ*ibpA*, Δ*ibpB*, Δ*STM1250* Δ*agsA* mutants are unaffected compared to WT by the presence of 6 mM H_2O_2. The initial growth rate (μ) of the lag phase, taken as the first 2 hours of growth, in the presence of 0 mM or 6 mM H_2O_2 was calculated. Data are the means of three separate experiments performed in duplicate with error bars representing SEM. Data was analysed by one-way ANOVA with Tukey's post-test, ns $p > 0.05$.

Due to the lack of significant sensitivity of the single mutants to 6 mM H_2O_2, it was proposed that the phenotype observed during growth of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was due to functional overlap between the four genes. Therefore, exposure of the multiple deletion mutants to 6 mM H_2O_2 may reveal functional overlap between the *ibpA*, *ibpB*, *STM1250* and agsA genes. Growth curves were conducted using the following multiple deletion strains: double (Δ*ibpAB*, Δ*STM1250*Δ*agsA*), triple (Δ*ibpAB*Δ*agsA*) and quadruple (Δ*ibpAB*Δ*STM1250*Δ*agsA*) mutants.

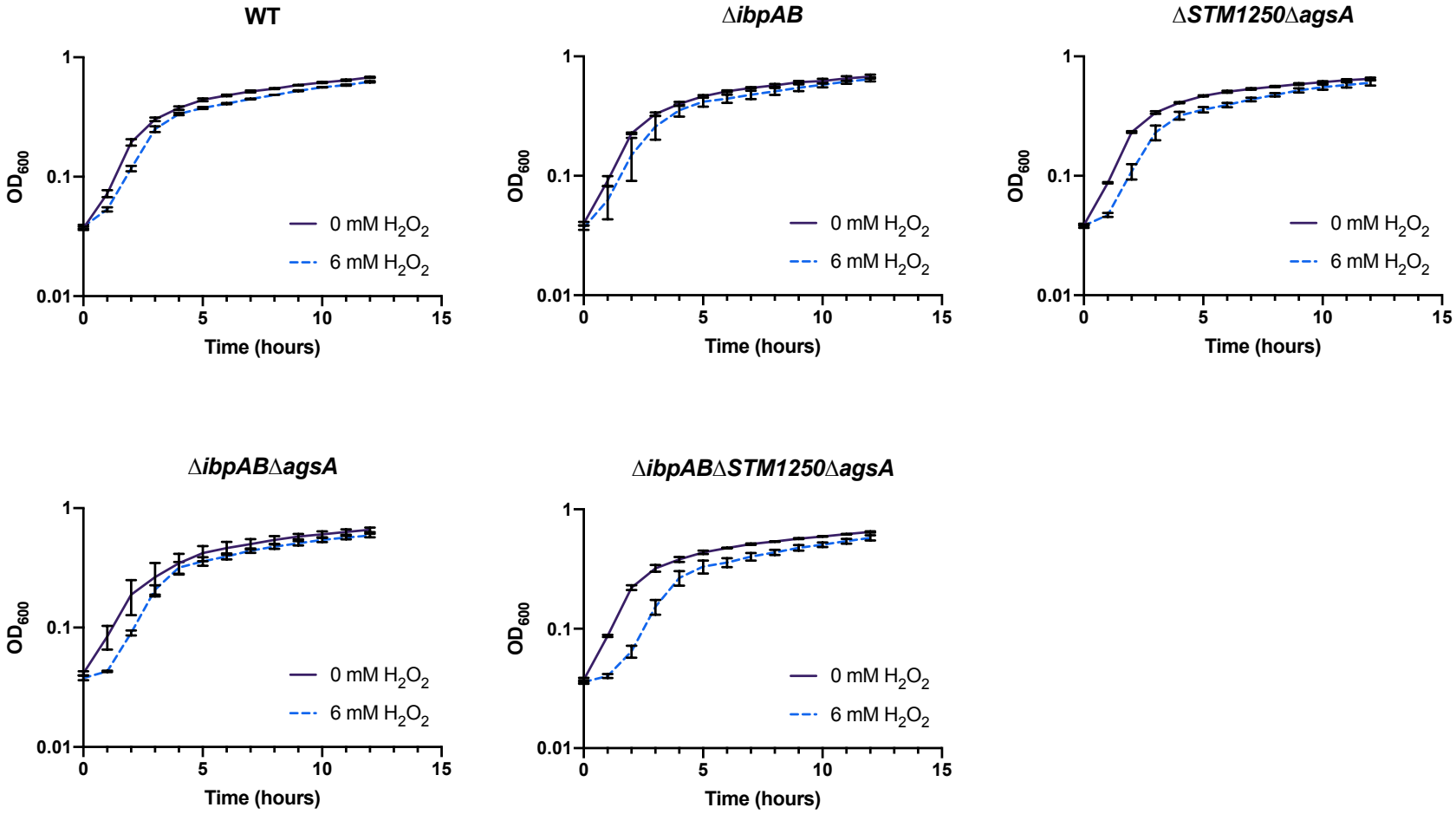


Figure 65. The lag phase of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is extended by 6 mM H_2O_2. Bacteria were grown in 24-well plates in LB containing 0 mM or 6 mM H_2O_2 in LB and incubated at 37 °C in a plate reader for 12 hours. OD_{600} readings were taken hourly. Data is representative of three separate experiments performed in duplicate and error bars are SEM.

Growth curve results presented in Figure 65 confirmed the initial results (Figure 62) in that the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant exhibits increased sensitivity to 6 mM H_2O_2 in LB with an extended lag phase. Initial growth of the Δ*STM1250*Δ*agsA* and Δ*ibpAB*Δ*agsA* mutants were also reduced, but not to the same extent as the quadruple mutant.

The initial growth rate of the bacteria grown with either 0 mM or 6 mM H_2O_2 was calculated. As shown in Figure 66 the initial growth rate was equal for all strains in the absence of H_2O_2. In contrast, in the presence of 6 mM H_2O_2, there was a significant reduction (approximately two-fold) between the initial growth rate of the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant. Furthermore, the growth rate of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly reduced compared to the Δ*STM1250*Δ*agsA* double mutant.

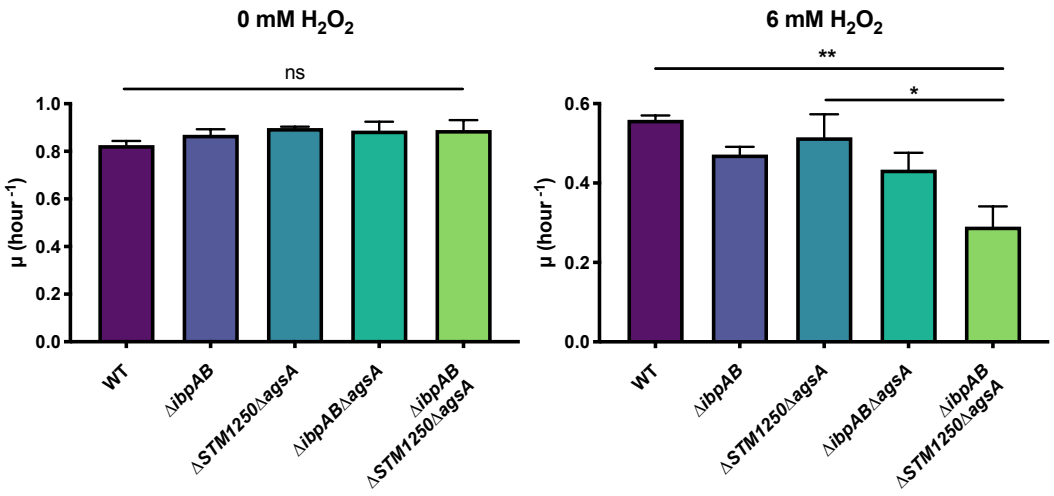


Figure 66. The lag phase of the Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant is extended during growth in the presence of 6 mM H_2O_2. The initial growth rate (μ) of the lag phase, taken as the first 2 hours of growth, in the presence of 0 mM or 6 mM H_2O_2 was calculated. Data are the means of three separate experiments performed in duplicate with error bars representing SEM. Data was analysed by one-way ANOVA with Tukey's post-test. * $p < 0.05$. ** $p < 0.005$, ns $p > 0.05$.

In order to verify that the presence of 6 mM H_2O_2 was responsible for the extended lag phase of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant, growth curves were also conducted in LB containing 6 mM H_2O_2 and bovine catalase. Bovine catalase is an

H_2O_2 scavenger and catalyses the breakdown of H_2O_2 into molecular oxygen (O_2) and water (H_2O) (Hébrard et al., 2009, van der Heijden et al., 2016).

Growth curves of the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant were performed in the presence of catalase only or catalase and 6 mM H_2O_2. In comparison to the results in Figure 65, there was no visible difference between the growth of *Salmonella* under these two conditions (Figure 67A). Moreover, this was illustrated by calculation of the initial growth rate as above (Figure 67B). Under each condition, there was no significant difference between the WT and quadruple mutant, therefore confirming that H_2O_2 treatment has a detrimental effect on the growth of the Δ*ibpAB*Δ*STM1250*Δ*agsA* strain.

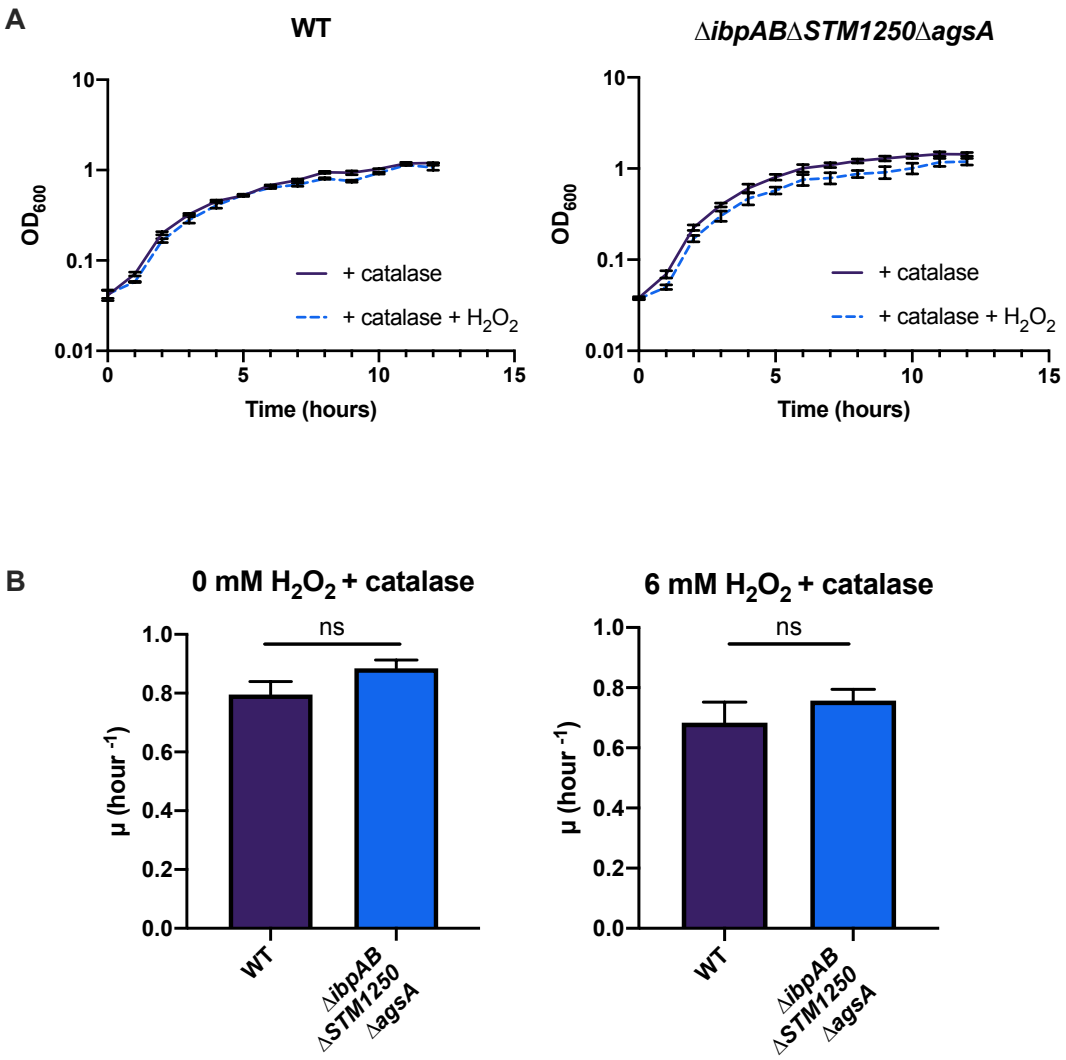


Figure 67. Bovine catalase restores growth of Δ*ibpAB*Δ*STM1250*Δ*agsA* to WT levels in the presence of 6 mM H_2O_2. Bacteria were grown in 24-well plates in LB containing 1000-4000 U/mL bovine catalase and either 0 mM or 6 mM H_2O_2 in LB and incubated at 37 °C in a plate reader for 12 hours. (A) OD_{600} readings were taken hourly. (B) The initial growth rate was calculated for the first 2 hours of growth in the presence of 0 mM or 6 mM H_2O_2. Data is representative of three separate experiments performed in duplicate and error bars are SEM. Data analysed by Student's *t*-test, ns $p > 0.05$.

To determine whether the LB growth media affected the potency of H_2O_2 towards *Salmonella* and whether ROS by-products were forming as a result of reactions between H_2O_2 and LB components (such as Fenton reactions with iron), growth curves were also conducted in minimal media (M9). WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* strains were grown in M9 containing either 0 mM, 1.5 mM,

3 mM or 6 mM H_2O_2. The quadruple mutant grew equally to the WT at 1.5 mM and 3 mM H_2O_2. However, in the presence of 6 mM H_2O_2 there was extremely limited growth of the Δ*ibpAB*Δ*STM1250*Δ*agsA* for the first 10 hours, although growth recovered and reached that of WT levels by 16 hours.

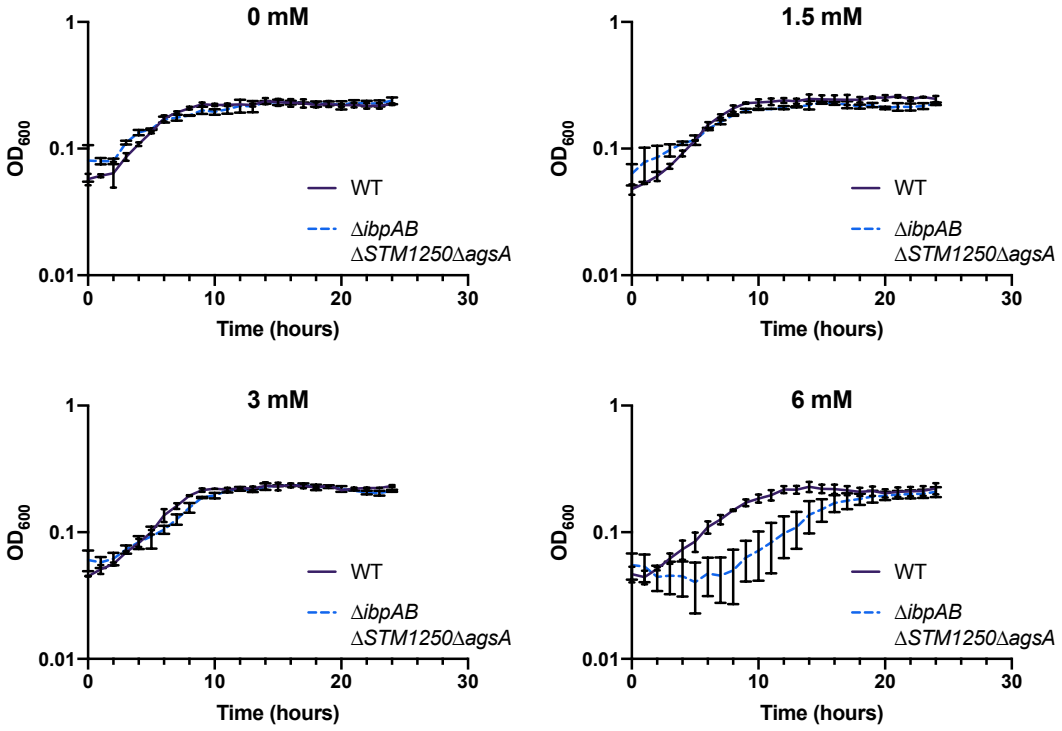


Figure 68. The Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant is more sensitive than WT to 6 mM H_2O_2, but is unaffected by 1.5 mM and 3 mM H_2O_2 during growth in M9 minimal media. Bacteria were grown in 24-well plates in M9 minimal media containing 0 mM or 6 mM H_2O_2 in LB and incubated at 37 °C in a plate reader for 12 hours. OD_{600} readings were taken hourly. Data is representative of three separate experiments performed in duplicate and error bars are SEM.

Continuing on the theme of functional redundancy, the growth curve studies were extended to the remaining combined deletion mutants (Δ*ibpAB*, Δ*STM1250*Δ*agsA* and Δ*ibpAB*Δ*agsA*) in M9 minimal media supplemented with 6 mM H_2O_2.

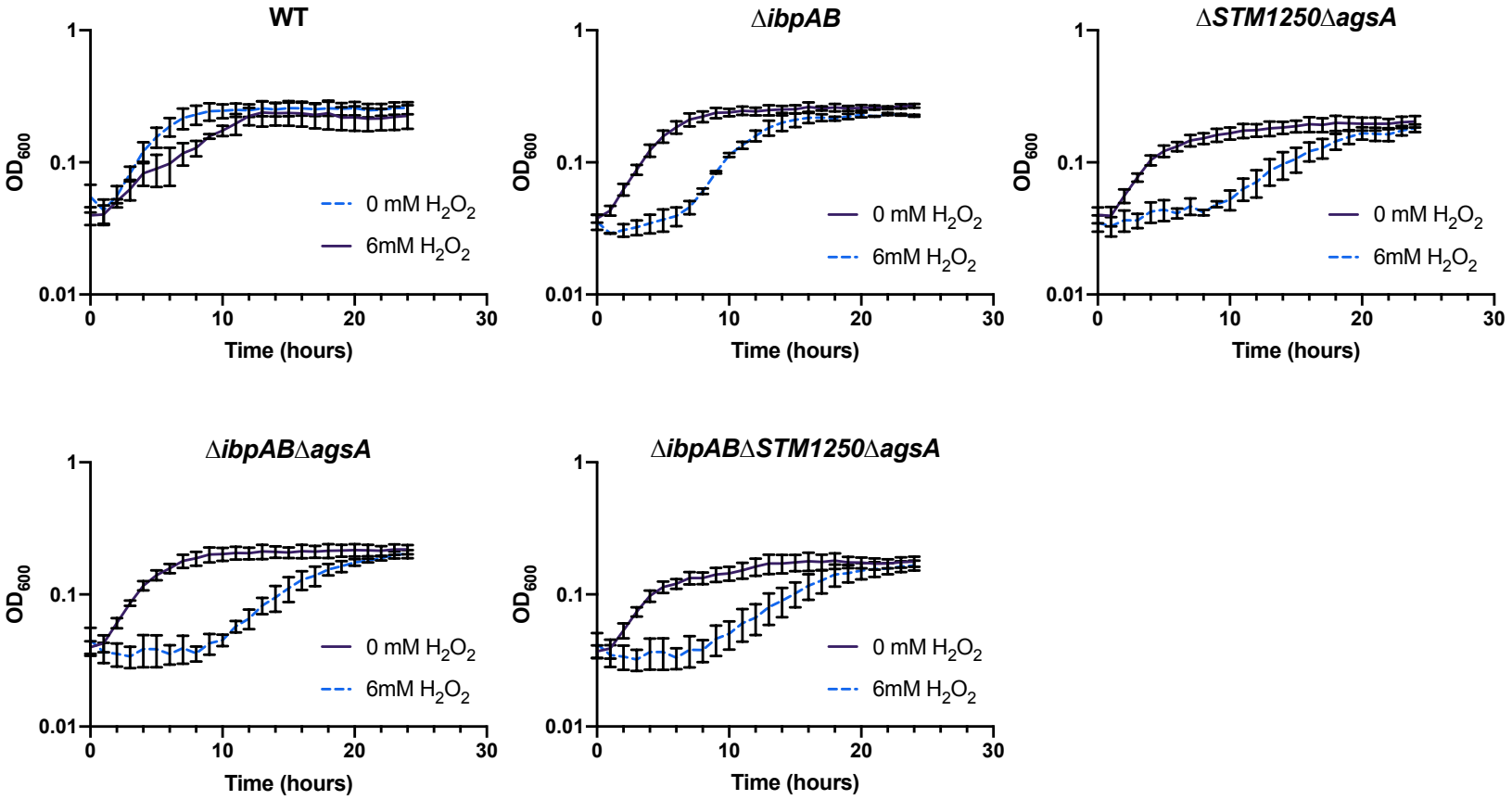


Figure 69. The lag phase of Δ*ibpAB*, Δ*STM1250*Δ*agsA*, Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* is extended by 6 mM H_2O_2 during growth in M9 minimal media. Bacteria were grown in 24-well plates in M9 minimal media containing 0 mM or 6 mM H_2O_2 in LB and incubated at 37 °C in a plate reader for 24 hours. OD_{600} readings were taken hourly. Data is representative of three separate experiments performed in duplicate and error bars are SEM.

In comparison to the growth curves conducted in LB, the addition of H_2O_2 to M9 minimal media caused a marked difference in the growth of all mutants tested, with the triple Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants displaying a highly extended lag phase (Figure 69). Lag phases of the four mutants tested were extremely long and ranged between 8 hours and 10 hours. However, beyond this extended lag phase, growth resumed in an exponential manner and the OD_{600} of bacteria grown with H_2O_2 were equivalent to those grown in the absence of H_2O_2 by 10 hours for WT and 16 – 18 hours for the four mutants.

4.2.9 The growth defect of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is limited to H_2O_2 oxidative stress and is not observed in the presence of alternative oxidants

In addition to H_2O_2, ROS production and oxidative stress can be induced by paraquat (methyl viologen) (Testerman et al., 2002). Moreover, compounds such as copper, potassium tellurite (K_2TeO_3) and indole have been shown to induce oxidative stress or an antioxidant stress response in bacteria (Garbe et al., 2000, Pérez et al., 2007, Matuszewska et al., 2008). To investigate whether the sensitivity of Δ*ibpAB*Δ*STM1250*Δ*agsA* was limited to H_2O_2-induced stress or whether alternative oxidants cause a similar response, growth curves were conducted in the presence of 30 mM paraquat, 0.5 µM K_2TeO_3 or 1 mM indole. The response of *Salmonella* to copper was investigated in Chapter 3 so will not be covered here.

4.2.9.1 Paraquat

In the literature, H_2O_2 and paraquat (methyl viologen) are the most commonly used oxidants during investigations of bacterial stress responses to oxidative stress. In the literature, *Salmonella* deletion mutants have been shown to be sensitive to paraquat during growth curve and disc diffusion assays (Humphreys et al., 1999, Testerman et al., 2002). Testerman et al. (2002) showed that survival of a *Salmonella rpoE* mutant is significantly reduced following a shock treatment in exponential phase of growth with 20 mM paraquat. In the same study, the survival of the *rpoE* mutant was significantly reduced in the presence of 4 mM H_2O_2. Based on these concentrations, *Salmonella* growth curves were conducted with 30 mM paraquat. Growth curves and analysis of the initial growth rates of the WT and mutants indicated that there is no difference in the growth of the strains tested and that 30 mM paraquat does not affect the growth of WT or the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant in this study (Figure 73).

4.2.9.2 Tellurite

Tellurium oxyanions (TeO_3^{2-}), including tellurite, are toxic to bacteria although some bacteria do exhibit resistance via the ability to reduce toxic TeO_3^{2-} to non-toxic elemental tellurium (Te^0) (Chasteen et al., 2009). It is understood that tellurite exerts its toxic effects through the oxidation of intracellular components and processes and through the generation of ROS, for example superoxide. A previous study in *E. coli* identified that the activity of the *ibpA* promoter is increased in the presence of K_2TeO_3 (Pérez et al., 2007) and this was linked to the production of superoxide radicals and the role of IbpA in *E. coli* for superoxide resistance (Kitagawa et al., 2000). Resistance to tellurite has been shown to be increased under anaerobic conditions and this was predicted to be due to a reduction in intracellular ROS production that usually arises as a by-product of aerobic respiration (Tantaleán et al., 2003, Pérez et al., 2007). A previous study investigating *Salmonella* resistance to tellurite identified that tellurite resistance genes were required for resistance to 0.5 μM K_2TeO_3, but the WT was unaffected by this concentration (Johnston, 2017).

In this study, growth curves were conducted aerobically in the presence of 0.5 μM K_2TeO_3. The results presented in Figure 71 show that the presence of K_2TeO_3 caused a minor reduction in the exponential phase of growth of all strains, however deletions of *ibpAB* and/or *STM1250* and *agsA* did not lead to any marked differences in growth, compared to the WT. Moreover, this was demonstrated by comparison of the initial growth rate of the WT and mutant strains (Figure 73).

4.2.9.3 Indole

Indole has been shown to promote the production of superoxide, an oxidative radical. Moreover, in a previous study *agsA* was shown to be upregulated by 1 mM and 4 mM indole (Nikaido et al., 2012). The sensitivity of the strains in this study were assessed at these concentrations. We found that in LB containing 4 mM indole, bacteria were unable to grow (data not shown). At 1 mM, growth occurred and there was no difference between initial growth rate or overall growth of the WT or mutant strains (Figure 73).

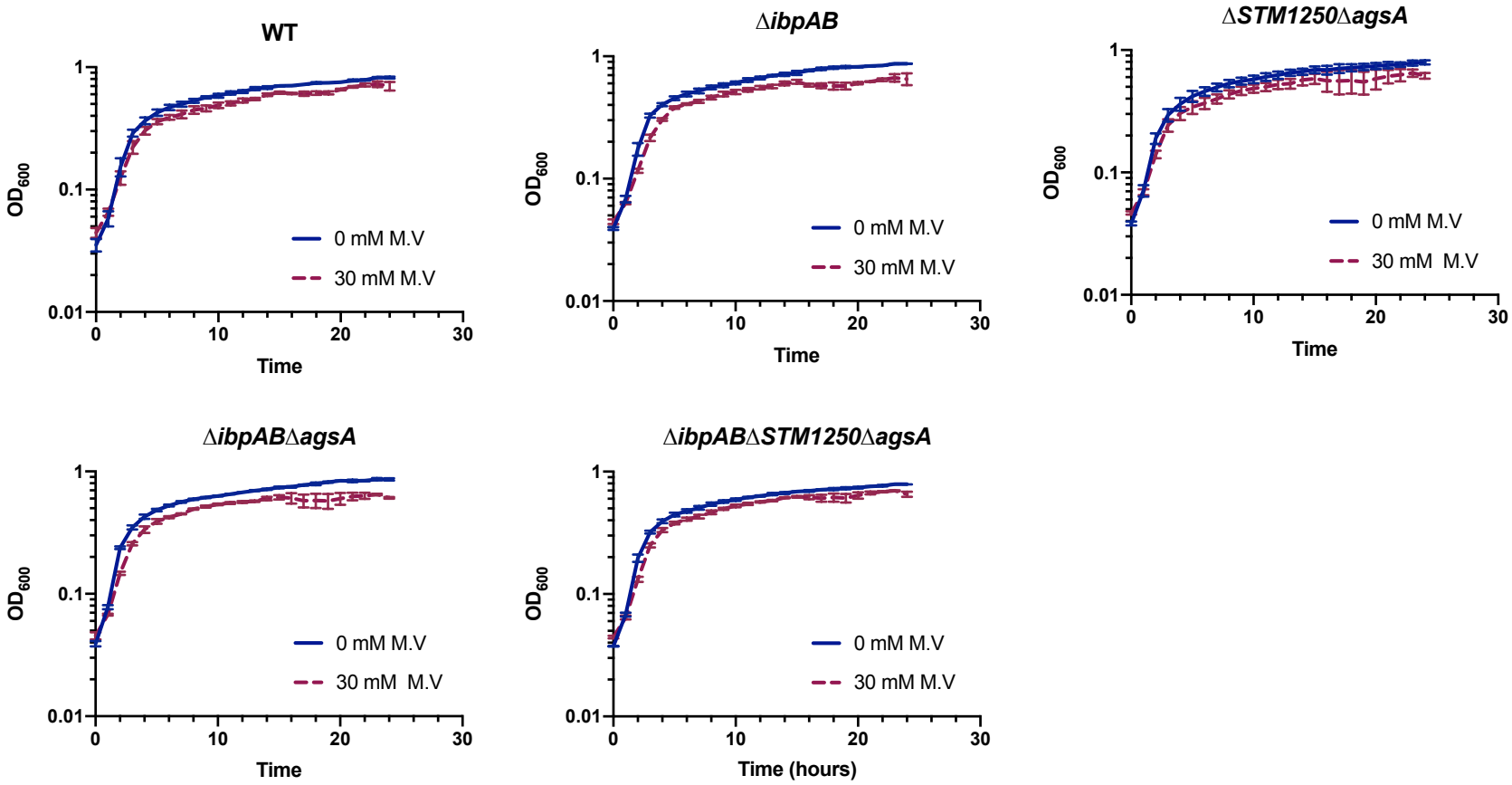


Figure 70. *ibpAB*, *STM1250* and *agsA* are not required for resistance to 30 mM methyl viologen (M.V). Bacteria were grown in 24-well plates in LB containing 0 mM or 30 mM methyl viologen aerobically at 37 °C for 24 hours. OD_{600} readings were taken hourly. Data points represent the means of three separate experiments performed in duplicate and error bars are SEM.

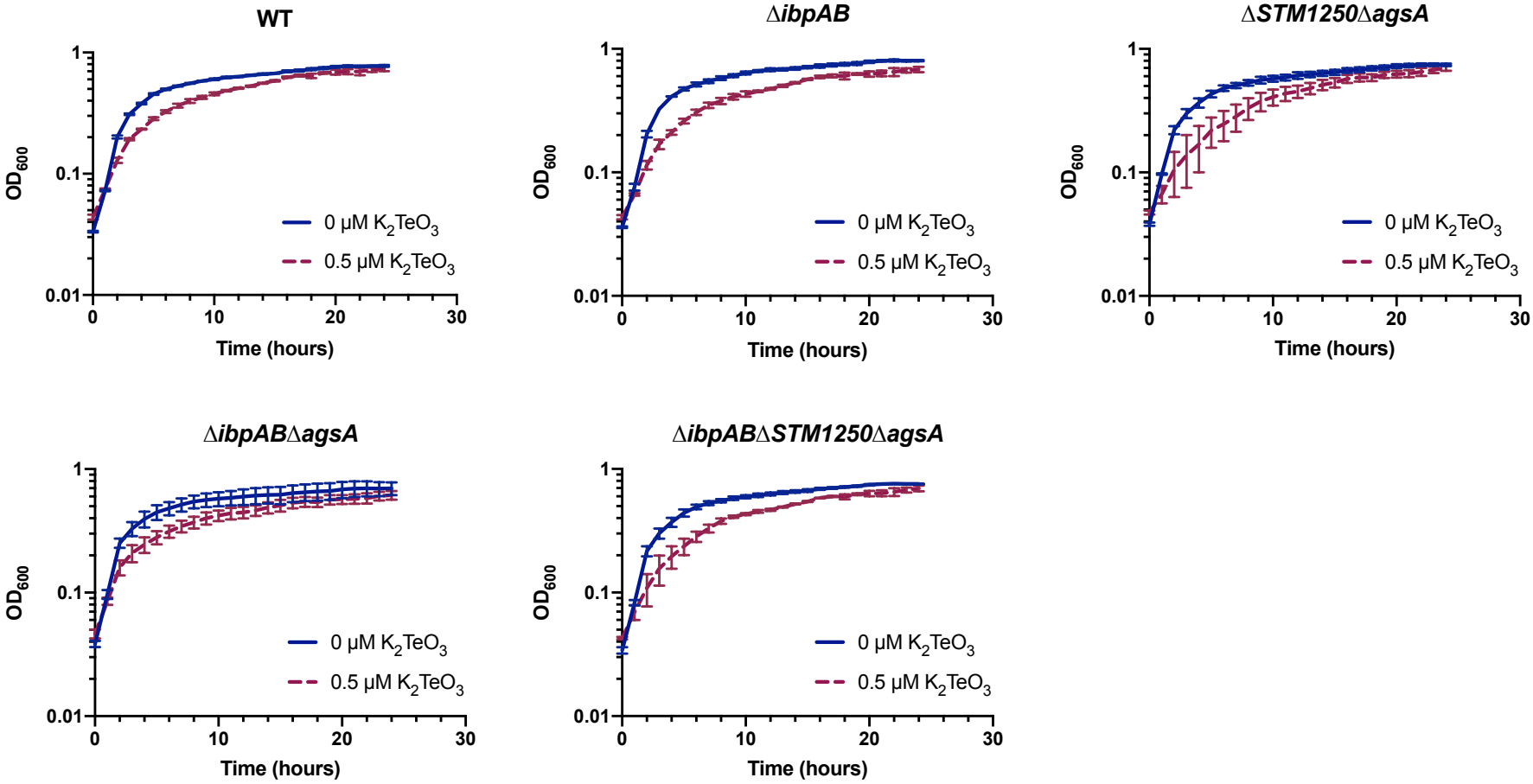


Figure 71. *ibpA*, *ibpB*, *STM1250* and *agsA* are not required for *Salmonella* resistance to 0.5 μM potassium tellurite (K_2TeO_3). Bacteria were grown aerobically in 24-well plates in LB containing 0 mM or 0.5 μM K_2TeO_3 at 37 °C for 24 hours. OD_{600} readings were taken hourly. Data points represent the means of three separate experiments performed in duplicate and error bars are SEM.

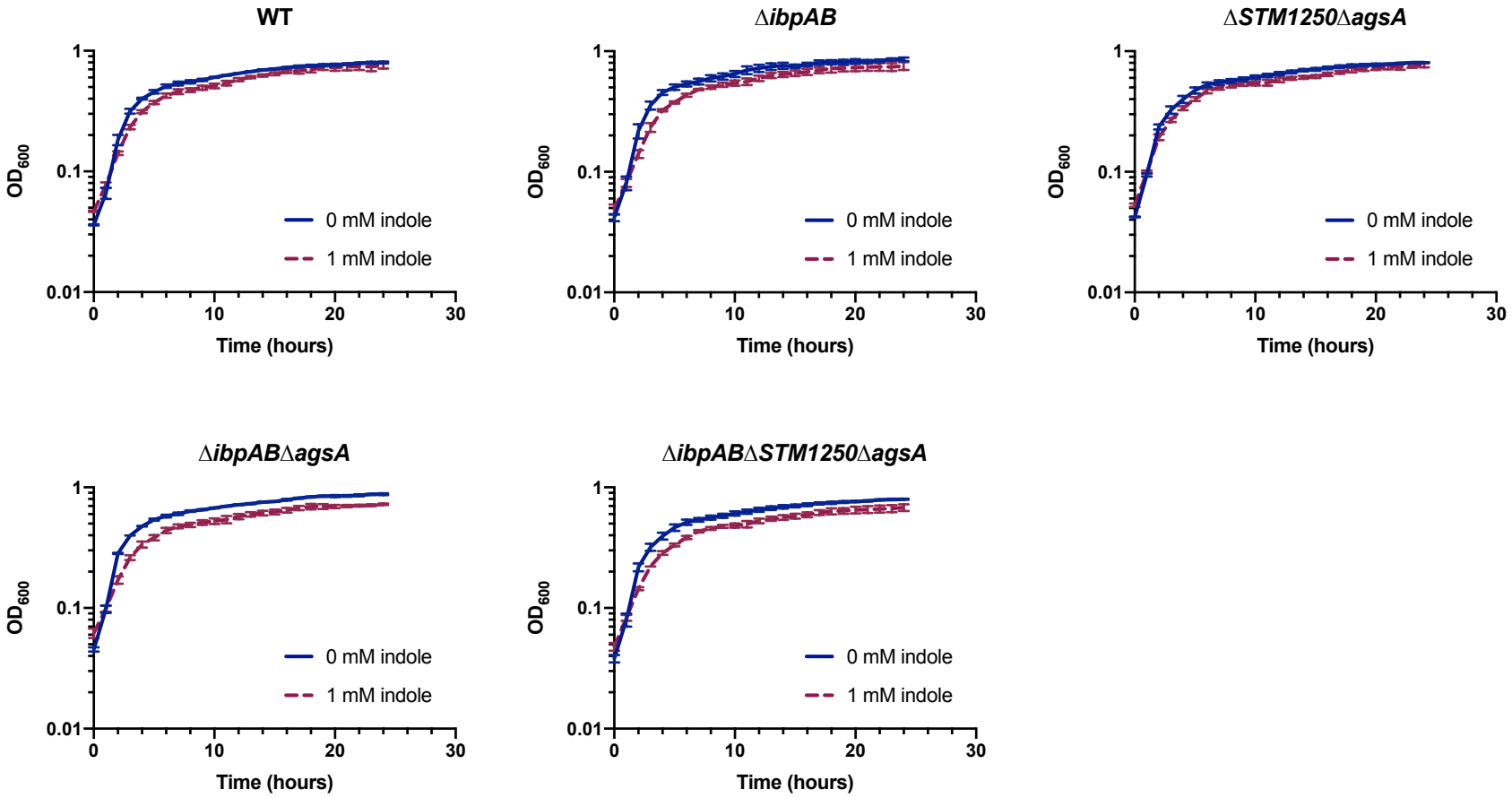


Figure 72. *ibpA*, *ibpB*, *STM1250* and *agsA* are not required for resistance to indole. Bacteria were grown aerobically in 24-well plates in LB containing 0 mM or 1 mM indole at 37 °C for 24 hours. OD_{600} readings were taken hourly. Data points represent the means of three separate experiments performed in duplicate and error bars are SEM.

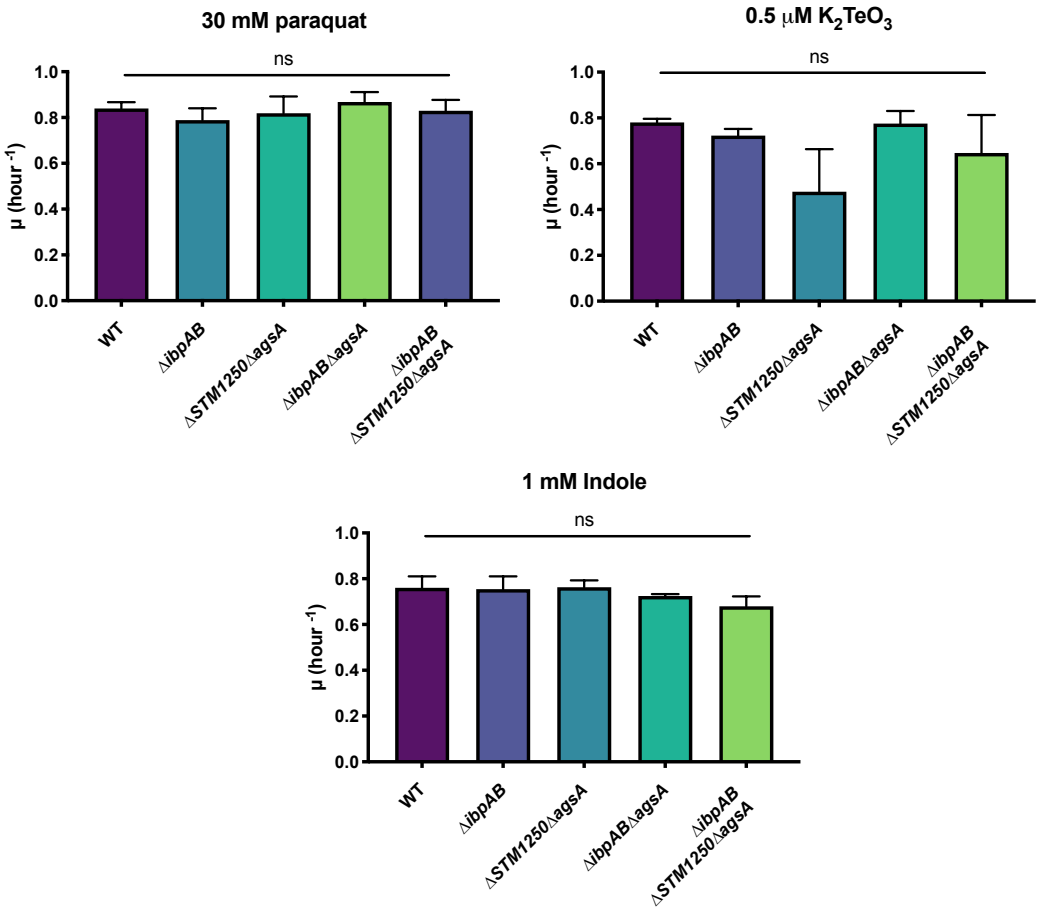


Figure 73. The initial growth rate of the Δ*ibpAB*, Δ*STM1250*Δ*agsA*, Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants is not significantly different to WT during growth in LB containing 30 mM paraquat, 0.5 μM potassium tellurite (K_2TeO_3) or 1 mM indole. The initial growth rate was calculated for the first 2 hours of growth. Data are the means of three separate experiments performed in duplicate and error bars represent SEM. Data was analysed by one-way ANOVA with Tukey's post-test. ns $p > 0.05$.

4.2.10 Functional overlap between IbpAB, STM1250 and AgsA during macrophage intracellular survival

The proximity of *STM1250* and *agsA* to the SPI-11 region coupled to the hypothesis that these genes may form a part of this island, indicated that they may play a role during host infection and in the survival against host-associated stresses. This was supported by data presented in this chapter and Chapter 3 in which the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was sensitive to H_2O_2-induced oxidative stress and polymyxin B and Δ*STM1250* is required for the adaptive acid tolerance response.

As a facultative intracellular pathogen, *Salmonella* thrives and grows within the intracellular space and the *Salmonella* containing vacuole. As described in the introduction to this chapter, macrophages are a site of ROS production and resistance to oxidative stress is an important contributor to the ability of *Salmonella* to cause infection. Furthermore, the acidified environment of the SCV presents additional stresses to the invading bacterium. In addition to the core family of bacterial catalases and alkyl hydroperoxide reductases, *rpoE* and σ^E-regulated genes are also important for resistance to oxidative stress and for intracellular survival (Testerman et al., 2002). Therefore, we hypothesised that this may include the *ibpAB, STM1250* and *agsA* group of genes. Previous studies have identified increased expression of *ibpA*, *ibpB*, *STM1250* and *agsA* during intracellular macrophage survival (Eriksson et al., 2003, Hautefort et al., 2008, Canals et al., 2019), however *Salmonella* deletion mutants of these genes have not been studied in this context.

4.2.10.1 IbpA, IbpB, STM1250 and AgsA are dispensable for survival in resting macrophages

Resting (un-activated) macrophages (RAW264.7) were infected with bacteria at a multiplicity of infection of 10:1 for 2 hours or 24 hours. Surviving CFU/mL were determined at each time point and fold change between these time points was calculated (Figure 74).

Overall, the mutants were not significantly attenuated after 24 hours of infection and the fold change between 2 and 24 hours was similar, with all strains exhibiting approximately a 10-fold increase in CFU/mL. Interestingly after 2 hours of infection, the intracellular CFU/mL of Δ*ibpAB*, Δ*ibpAB*Δ*agsA* and Δ*ibpAB*Δ*STM1250*Δ*agsA*, notably all mutants lacking the *ibpAB* gene pair, were significantly lower than that of WT (Figure 74).

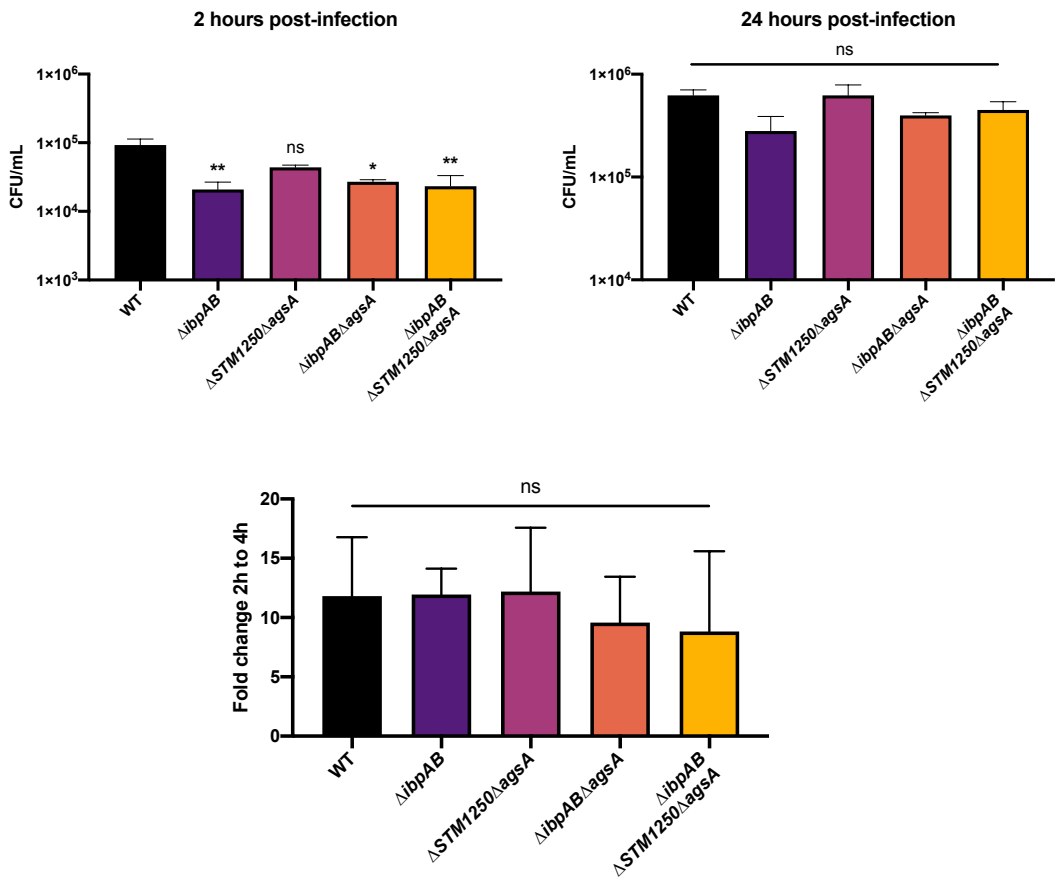


Figure 74. Intracellular CFU/mL of mutants lacking *ibpA* and *ibpB* are significantly reduced after 2 hours infection but there is no significant attenuation after 24 hours infection of RAW264.7 macrophages. Macrophages were infected with bacteria at a multiplicity of infection of 10:1 and incubated for the duration shown. Data are the means of three separate experiments performed in duplicate and error bars show SEM. Data analysed by one-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$ and ** $p < 0.005$.

4.2.10.2 The Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is attenuated in IFN-γ activated macrophages

IFN-γ activation of macrophages increases their phagocytic activity and production of ROS and RNS, thus producing more aggressive macrophages (Ingram et al., 2017). Macrophages were activated with IFN-γ 22 hours prior to infection. For infection, macrophages were inoculated with bacteria at a MOI of 10:1. As above, macrophages and bacteria were incubated for 2 hours or 24 hours and surviving, intracellular CFU/mL were determined. In addition, fold change of intracellular CFU/mL between 2 hours and 24 hours was calculated (Figure 75).

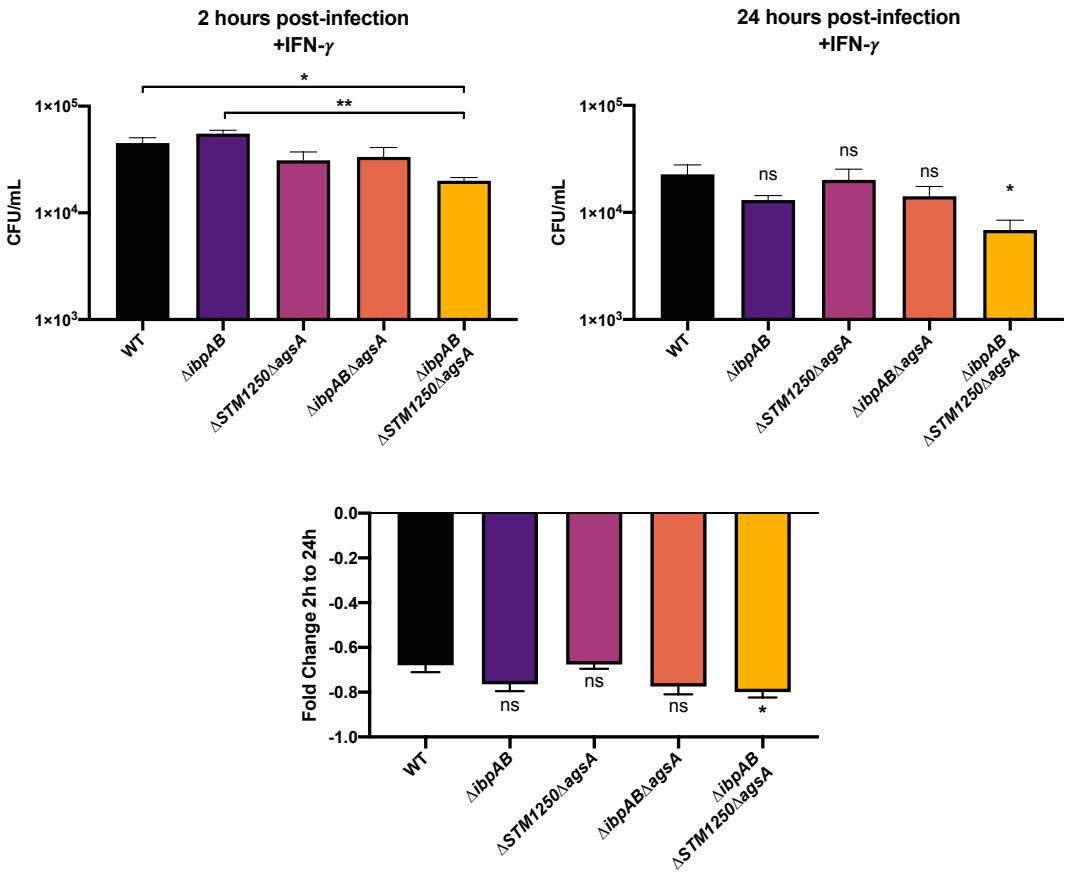


Figure 75. The Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is significantly attenuated in IFN-γ activated RAW264.7 macrophages after 2 hours and 24 hours of infection. Macrophages were infected with bacteria at a multiplicity of infection of 10:1 and incubated for the duration shown. Data are the means of three separate experiments performed in duplicate and error bars show SEM. Data analysed by one-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$ and ** $p < 0.005$.

Survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly reduced compared to WT at 2 hours and 24 hours post-infection while the survival rates of the remaining mutants were unchanged. In addition, after 2 hours of infection the number of CFU/mL of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant were significantly lower than that of the Δ*ibpAB* double mutant. This may indicate a potential role of STM1250 and/or AgsA in compensating for the loss of IbpA and IbpB, supporting the hypothesis of the existence of functional overlap between these four proteins during survival against stress. Although survival of all mutants lacking *ibpA* and *ibpB* was reduced after a 2 hour infection of resting macrophages, this was not the case for IFN-γ activated cells;

only survival of the quadruple mutant was reduced, supporting the hypothesis of functional overlap between IbpA, IbpB, STM1250 and AgsA in these conditions.

In comparison to the proliferation of *Salmonella* in resting macrophages, in IFN-γ activated cells the number of surviving bacteria reduced between the 2 hour and 24 hour incubation period. Moreover, the fold change reduction in CFU/mL of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly different to that of the WT, although as can be seen in Figure 75, the fold change of the Δ*ibpAB* and Δ*ibpAB*Δ*agsA* mutants were also reduced in comparison to WT but were not statistically different.

4.2.10.3 Survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is recovered by inhibition of the macrophage NADPH oxidase

Coupling the reduced survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant in IFN-γ-activated macrophages and its increased sensitivity of to H_2O_2, we hypothesised that the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is limited in its ability replicate in IFN-γ macrophages due to the high concentration of ROS within this environment. In order to investigate this, macrophages activated with IFN-γ were treated with apocynin (acetovanillone, 4-hydroxy- 3-methoxyacetophenon, Sigma), an inhibitor of the macrophage NADPH-oxidase (Vejražka et al., 2005).

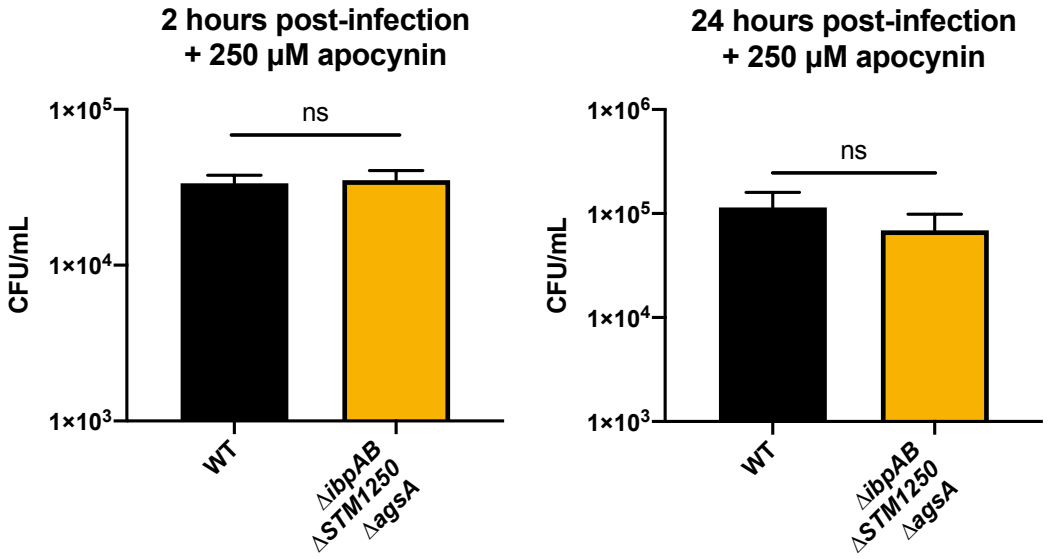


Figure 76. Treatment of RAW264.7 macrophages with 250 µM apocynin restores survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant to WT levels after 2 hours and 24 hours of infection. Macrophages were infected with bacteria at a multiplicity of infection of 10:1 and incubated for the duration shown. Apocynin was added at a final concentration of 250 µM at 0 hours, 2 hours and 8 hours post-infection. Data are the means of three separate experiments performed in duplicate and error bars show SEM. Data analysed by Student's *t*-test, ns $p > 0.05$.

Following infection of apocynin-treated macrophages, the surviving CFU/mL of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant were restored to WT levels at both 2 hours and 24 hours.

Figure 77 presents a summary of the CFU/mL for both strains under all intracellular survival conditions tested in this thesis. It can be observed that apocynin treatment of IFN-γ treated macrophages led to increased CFU/mL of both the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant, compared to IFN-γ treatment only. However, apocynin treatment did not completely restore CFU/mL to that of infection of resting macrophages (Figure 77).

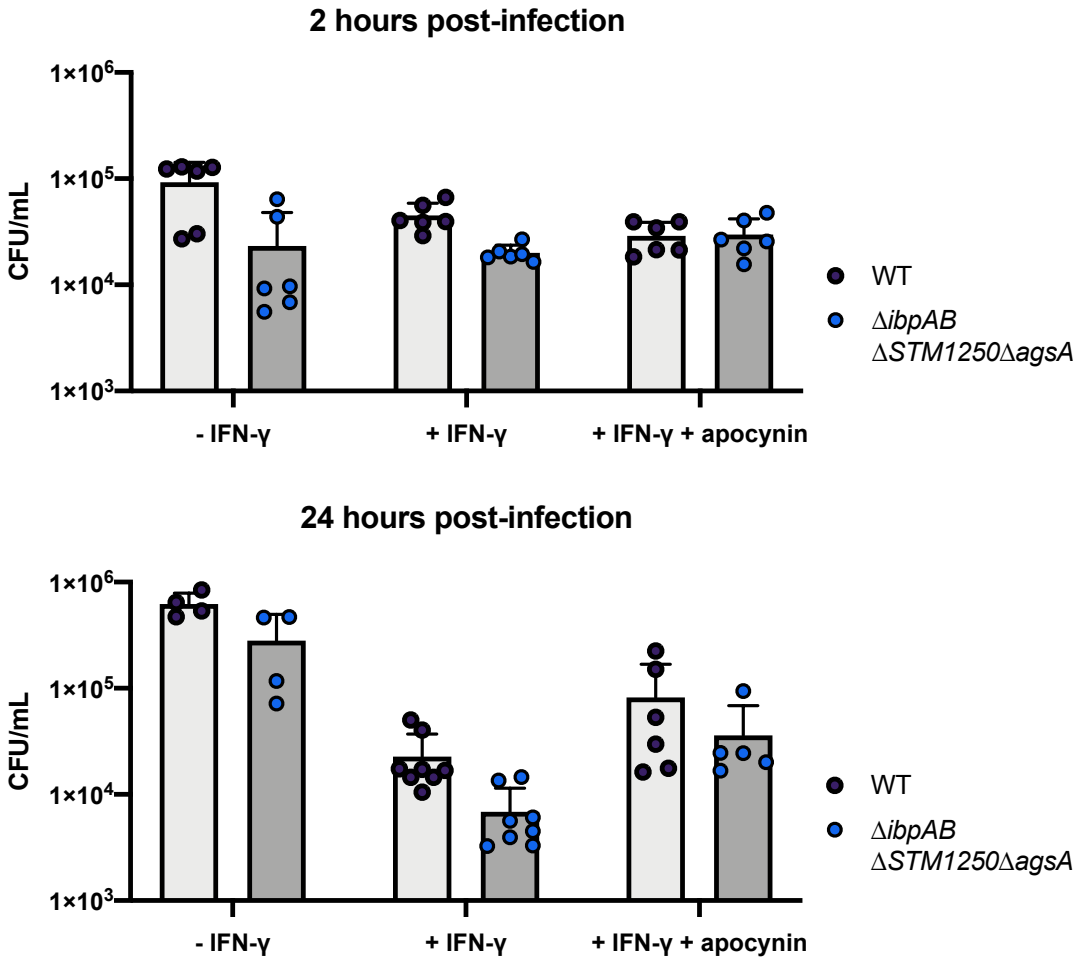


Figure 77. A summary of WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* survival in resting or activated macrophages +/- the NADPH oxidase inhibitor apocynin. The data represent a meta-analysis from all gentamicin protection assays reported in this thesis. Assays are reported in this figure as an overall summary but were performed at different times.

4.2.10.4 Overexpression of STM1250 does not fully restore Δ*ibpAB*Δ*STM1250*Δ*agsA* survival to WT levels following infection of IFN-γ activated macrophages

The reduced survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* quadruple mutant, but the lack of significant phenotype for the other mutants tested, suggests that the additive deletion of the four genes contributes to this phenotype. With the aim of further investigating functional overlap, the quadruple mutant was complemented with STM1250. The *STM1250* gene was cloned into an arabinose-inducible overexpression vector in order to determine whether overproduction of STM1250 leads to a restoration of survival of the mutant to WT levels.

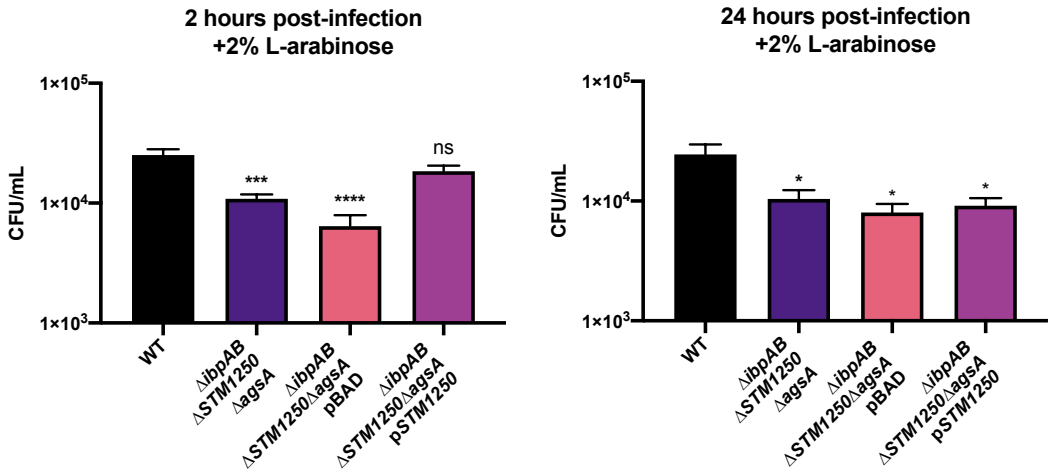


Figure 78. Overexpression of STM1250 in the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant partially restores survival to WT levels after 2 hours but not 24 hours of infection. Macrophages were infected with bacteria at a multiplicity of infection of 10:1 and incubated for the duration shown. L-arabinose was added to a final concentration of 2% at 0 hours, 2 hours and 8 hours post-infection. Data are the means of three separate experiments performed in duplicate and error bars show SEM. Data analysed by one-way ANOVA with Tukey's multiple comparisons test, ns $p > 0.05$, * $p < 0.05$, *** $p < 0.0005$ and **** $p < 0.0001$.

Overexpression of STM1250 in the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant strain partially restored survival to WT levels after 2 hours of infection and the Δ*ibpAB*Δ*STM1250*Δ*agsA* with empty pBAD vector control strain exhibited the same phenotype as the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant, confirming that this restoration of

survival could not be attributed to the addition of the pBAD plasmid. However, after 24 hours of infection overexpression of STM1250 did not restore the intracellular CFU/mL of the quadruple mutant to WT levels.

4.2.11 Proposed PhoPQ regulation of STM1250

Previous comparison of the PhoPQ regulon in *E. coli* and *Salmonella* identified multiple *Salmonella* specific genes with a conserved 5' PhoP binding motif (Monsieurs et al., 2005). The Monsieurs et al. (2005) study identified that a PhoP binding motif is located upstream of *STM1250.* As described in Chapter 1, PhoPQ is responsible for polymyxin B resistance and is also involved in intramacrophage survival and acid tolerance. Proposed regulation of *STM1250* by the PhoPQ TCS was investigated by qRT-PCR.

4.2.11.1 Regulation of STM1250 under polymyxin B treatment

Since polymyxin B is an inducer of the PhoPQ two component system, RNA was prepared from three biologically independent samples of the WT or Δ*phoP* mutant grown in the presence or absence of polymyxin B. It was verified that expression (C_T values) of the housekeeper genes, *ampD* and *gyrB*, are unchanged by the presence of polymyxin B.

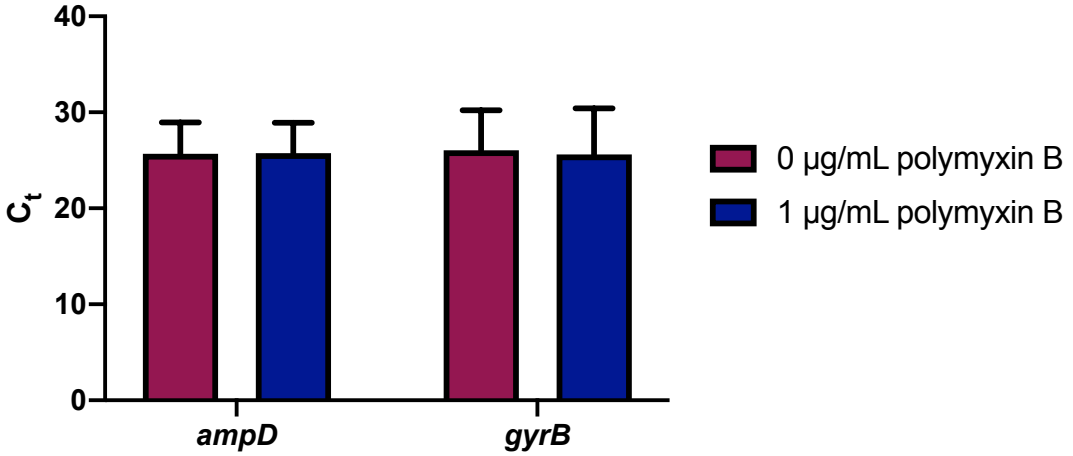


Figure 79. Ct values for the housekeeping genes *ampD* and *gyrB* remain unchanged by polymyxin B treatment. Data represents the means of the raw Ct values obtained from three separate qRT-PCR experiments. Error bars are SEM.

A previously identified PhoP-activated gene, *pagC*, was employed as a control to confirm that the PhoPQ system was being induced in the conditions in this study.

pagC and *STM1250* mRNA fold change in the WT and Δ*phoP* mutant, in the presence of polymyxin B (PhoPQ inducing conditions), was calculated using the $2^{-\Delta\Delta Ct}$ method, relative to polymyxin B free controls.

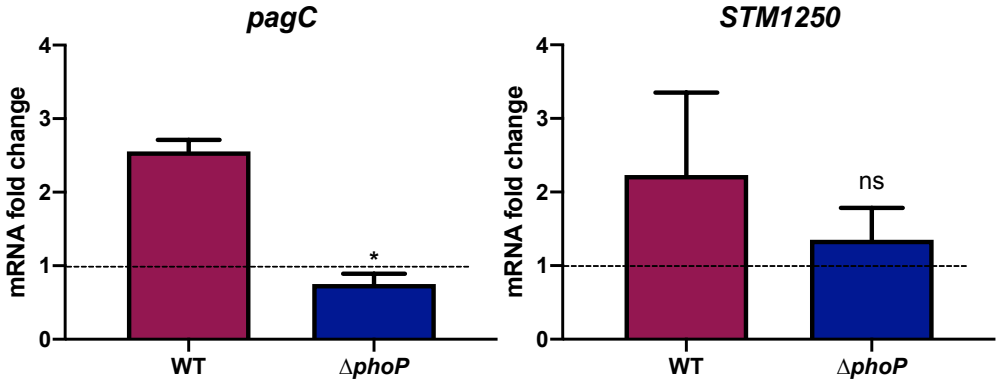


Figure 80. *STM1250* expression is not significantly altered in a Δ*phoP* mutant compared to WT in polymyxin B treated cells. *Salmonella* WT and Δ*phoP* mutant were exposed to 1 μg/mL polymyxin B or left untreated for one hour. Expression of *pagC* and *STM1250* was determined by qRT-PCR and mRNA fold change of polymyxin B treated cells was calculated using the $\Delta\Delta C_T$ method, relative to the untreated control. Data are the means of three separate RNA preparations and error bars show SEM. Data analysed by Student's *t*-test, * $p < 0.05$ and ns $p > 0.05$.

A significant reduction in mRNA fold change of *pagC* was confirmed between WT and ΔphoP; as expected *pagC* was upregulated in WT cultures and downregulated in Δ*phoP* cultures in the PhoPQ inducing conditions. *STM1250* was upregulated in the WT in the presence of polymyxin B, supporting data presented in Chapter 3 and a proposed role in polymyxin B resistance. Expression of *STM1250* in the Δ*phoP* mutant was reduced in comparison to WT (2-fold increase in *STM1250* expression in WT treated cells vs a fold change close to 1 in Δ*phoP* treated cells); however, statistical analysis did not confirm this to be a significant difference. Therefore, it cannot be statistically concluded that *STM1250* is regulated by PhoP on the basis of the current data.

4.3 Discussion

The aim of this chapter was to investigate the involvement of IbpA, IbpB, STM1250 and AgsA in the survival of *Salmonella* against host-associated stresses, with a focus on the acid tolerance response and oxidative stress. σ^E has been attributed to *Salmonella* acid resistance but the molecular mechanisms or precise genes involved are poorly characterised. In terms of oxidative stress, a small number of studies exist describing a role for IbpAB in *E. coli* resistance to copper- (Matuszewska et al., 2008) and paraquat-induced (Kitagawa et al., 2000) oxidative stress but to date, these studies have not been extended to *Salmonella*. Environmental acidification and oxidative stress are two important mechanisms utilised by phagocytes with the aim of eliminating invading pathogens. In this chapter, we sought to determine the contribution of IbpA, IbpB, STM1250 and AgsA to *Salmonella* acid tolerance, oxidative stress resistance and intra-macrophage survival.

4.3.1 STM1250 is important in the *Salmonella* ATR

In addition to the central regulators of the ATR (RpoS, Fur, PhoPQ and OmpR/EnvZ), *rpoE* has been shown to be induced by acid shock in *Salmonella* (Muller et al., 2009). Despite this, evidence in the literature for RpoE-regulated proteins and their roles in the acid shock response is extremely limited. Acid shock response regulator genes have been proposed to provide good targets as live attenuated *Salmonella* vaccines (Rychlik and Barrow, 2005); however, *rpoE* mutants are highly attenuated and do not provide protection against subsequent WT challenge (Humphreys et al., 1999). With this in mind, *rpoE*-regulated genes may provide routes for alternative targets and attenuated strains.

To investigate the contribution of the *ibpAB*, *STM1250* and *agsA* genes to the ATR, the survival of the WT and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant following acid shock (HCl, pH 3.0), either with or without a 2 hour adaption period (HCl, pH 4.4), was assessed. The results revealed a significant reduction in survival of the mutant after 30 mins exposure, in adapted cultures only. These initial results indicated that the genes in the quadruple deletion mutant are required for *Salmonella* to tolerate an extreme acidic environment and mount an adaptive ATR. Further ATR experiments performed in the same manner in fact identified that *STM1250* solely contributed to this phenotype. Since previous phenotypes associated with an *STM1250* deletion also required the deletion of the sHsps *ibpAB* and *agsA* (Chapter 3) (Hews et al., 2019b), this was the first identification of an *STM1250* specific role in *S*. Typhimurium.

The ∆*STM1250* mutant was also sensitive to inorganic acid shock (HCl) in the exponential phase ATR. Moreover, the same response was observed when *Salmonella* was subjected to acetic acid. ATR assays in acetic acid were performed using a less acidic pH than the HCl assays; *Salmonella* was unable survive in adaptation media adjusted to pH 4.4 with acetic acid. In agreement with the increased sensitivity observed in this thesis, previous studies have also observed increased killing of *Salmonella* in acetic acid media at pH 4 (Burin et al., 2014). In all cases, the greatest difference between WT and ∆*STM1250* rate of survival was observed during the first 30 mins of acid shock, suggesting that STM1250 is required by *Salmonella* in the initial stages of acid exposure and this is not limited to the growth phase or acid used. In the presence of HCl, this requirement appears to be specific to the acid shock pH 3.0 exposure, since CFU/mL of the WT and mutants were at equivalent levels following the 2 hour adaptation period at pH 4.4. In contrast, in the presence of acetic acid there was a significant reduction in CFU/mL of the ∆*STM1250* strain compared to WT after the 2 hour adaptation. Acetic acid is highly liposoluble and with a small molecular weight can traverse bacterial membranes easily (Fernandez et al., 2009, Álvarez-Ordóñez et al., 2010). It is reported to result in the greatest inhibition of *Salmonella* growth/survival, ahead of lactic acid, citric acid and hydrochloric acid (in order of decreasing potency) (Jung and Beuchat, 2000, Álvarez-Ordóñez et al., 2010). Once within the cytoplasm, organic acids dissociate and reduce cytoplasmic pH leading to intracellular protein damage. Therefore, the reduction in survival of the ∆*STM1250* mutant suggests that STM1250 may function either to increase/maintain intracellular pH or to respond to low pH induced protein damage.

Previous analysis of the *Salmonella* global ATR transcriptome supports the data presented in this thesis; *STM1250* was reported to be upregulated following 1 hour adaptation at pH 4.4, but this upregulation was not observed following subsequent acid shock for 1 hour at pH 3.0 (Ryan et al., 2015); we did not observe a difference in survival between the WT and ∆*STM1250* mutant after 1 hour exposure to pH 3.0. In contrast, Kröger et al. (2013) reported that expression of *ibpA*, *ibpB*, *STM1250* and *agsA* are downregulated or unchanged in response to acid shock at pH 5.8 or pH 3.0. However, the acid shock assays in the in Kröger et al. (2013) were not preceded by an adaptation period. Our data indicates that STM1250 is important in the adaptation phase, to increase survival at low pH but does not contribute to survival of unadapted *S.* Typhimurium. The requirement of STM1250, and its precise involvement in adaptation to acid shock, should certainly be further elucidated by investigating

STM1250 expression during incubation at pH 4.4 and at each time point during pH 3.0 acid shock via qRT-PCR.

STM1250 has not been phenotypically associated with the *Salmonella* ATR response prior to this study. In contrast to the Ryan et al. (2015) study, Cao et al. (2019) reported upregulation of *STM1250* following exposure of *Salmonella* to pH 3.1 for 1 hour, with prior adaptation at pH 4.4. Interestingly, in the same study all three sHsps (*ibpA, ibpB* and *agsA*) were also reported to be upregulated. The effect of media composition should be emphasised here; the Cao et al. (2019) study performed acid shock assays in tryptic soy broth (TSB) and the acid used for adjustment of pH was not stated, whereas Ryan et al. (2015) utilised a minimal E glucose medium (more closely similar to the media used in this thesis) and the pH was also adjusted with HCl. Therefore, it would be beneficial to determine whether the phenotypes reported here are replicated in a rich medium such as LB or TSB. It should also be noted that in such media, the presence of amino acids would also induce the homeostatic mechanisms of the lysine/arginine decarboxylase systems (Díaz and Ricke, 2004).

Complementation of the Δ*STM1250* mutant was achieved via IPTG-induced overexpression of *STM1250* from the pET-Duet1 vector. Interestingly, overexpression of STM1250 in both WT and Δ*STM1250* strains significantly increased survival beyond that of the WT. When overexpression of STM1250 is induced, expression is sustained which may explain the increase in survival at the later time points. The results suggest that native expression of *STM1250* peaks during the first 30 mins since after this the survival of the WT and Δ*STM1250* mutant are equal, but as indicated above this requires confirmation by qRT-PCR. For complementation, STM1250 was overexpressed during adaptation at pH 4.4 and acid shock at pH 3.0. Limiting overexpression to the acid shock period only may confirm the importance of STM1250 during the adaptation phase. As noted in the previous Chapter, a limitation of this work is the concentration of IPTG used to overexpress the STM1250 protein. A 1 mM final concentration of IPTG is relatively high and in future work, it may be beneficial to investigate optimal overexpression conditions with different concentrations of IPTG. This would ensure that only the presence of acid stress is affecting bacterial growth, instead of a cumulative effect of environmental stress and the metabolic stress associated with protein overproduction.

As a result, in addition to inducible overexpression, the data would be further supported by phenotypic complementation using a low-copy non-inducible vector, to confirm whether native expression of *STM1250* restores survival of the Δ*STM1250* mutant to WT levels. Finally, whilst this thesis has focussed on the ATR in *S*

Typhimurium, other enteric Gram-negative pathogens must also be able to resist the acidic environment of the stomach. Since *E. coli* lacks the *STM1250* gene but does encode the potentially functionally overlapping IbpA and IbpB, it would be interesting to determine whether heterologous overexpression of STM1250 enhances *E. coli* WT and Δ*ibpAB* resistance to acid.

As indicated throughout this chapter, the *Salmonella* ATR is affected by the type of acid used and the composition of the assay media. Therefore, although previously characterised in the literature, we assessed the ATR of the Δ*rpoE* and Δ*phoP* mutants in the glucose minimal media (adjusted to pH 4.4 (for adaptation) and pH 3 (for acid shock) with HCl) used in this study. Interestingly, despite being associated with an inorganic acid ATR and induced in acidic conditions, the Δ*phoP* mutant behaved in an identical manner to WT and survival was unaffected following acid shock. This suggests that despite the presence of a PhoP motif upstream of *STM1250*, *STM1250* is not regulated by PhoPQ in acidic conditions. However, to confirm this, and to further investigate individual contributions of the PhoPQ TCS components to the ATR, it would be beneficial to investigate the response of the Δ*phoQ* and Δ*phoP*Δ*phoQ* mutants. In contrast, the Δ*rpoE* mutant was significantly affected by acidification of the adaptation and shock media and there were no viable cells remaining after 60 min incubation at pH 3.0. Since acid shock activates the *rpoE* regulon in a non-canonical manner it would also be interesting to determine whether *rpoE* is fully activated in the Δ*STM1250* mutant (Muller et al., 2009).

The importance of the *Salmonella* ATR and identifying the precise mechanisms involved in response to varying acids and growth conditions should not be limited to one *Salmonella* serovar. The experiments in this thesis have focussed on the NTS serovar *S.* Typhimurium; however, *S.* Enteritidis is prevalent in the food chain and is one of the major contributors to foodborne *Salmonella* infections. Furthermore, systemic serovars like *S.* Typhi, are especially adapted to survival in the host environment. Interestingly, the amino acid decarboxylase systems (lysine and arginine) are not conserved in systemic *Salmonella* serovars, indicating that the precise acid resistance mechanisms may be different among serovars (Viala et al., 2011). Therefore, it should be of primary interest to extend the ATR studies in this chapter into other serovars. Finally, although the main focus of this chapter is *Salmonella* survival in the host, food preservation and sterilisation treatments are also highly dependent on the use of acid, salt and heat. A further discussion of the acid shock sensitivity of Δ*STM1250* in the context of food security will be discussed in

more detail in Chapter 6 (General Discussion) in order to contextualise the results alongside the temperature sensitive phenotypes associated with IbpAB and AgsA.

4.3.2 Functional overlap between IbpAB, STM1250 and AgsA is observed in response to hydrogen peroxide treatment

Oxidative stress is routinely encountered by bacteria; endogenous ROS are produced as a by-product of aerobic respiration and bacteria are able to tolerate these levels of ROS without significant damage or changes to intracellular processes (Imlay, 2019). However, during colonisation of a host, *Salmonella* must also be able to survive the harsh oxidising environments that arise as part of the host's immune defences to infection.

Despite their description as sHsps, as described in the previous chapter this family of proteins have been shown to rescue bacteria from multiple stressors, not limited to heat. One such example is oxidative stress and links between the heat shock and oxidative stress response have been demonstrated previously; Marcén et al. (2017) reported that ROS are present in *E. coli* heat treated cells and the addition of antioxidant to the media during heat treatment led to protection of *E. coli* against heat shock. Moreover, in *E. coli* overexpression of the sHsps IbpA and IbpB provides resistance to paraquat (Kitagawa et al., 2000) and *E. coli* Δ*ibpAB* deletion mutants are sensitive to copper-induced oxidative stress (Matuszewska et al., 2008). However, the contribution of IbpAB to *Salmonella* oxidative stress resistance has not been studied to the same extent. The σ^E regulon in *Salmonella* is required for murine infection and is highly important for survival during growth in the presence of H_2O_2 and paraquat oxidants (Testerman et al., 2002). We hypothesised that the σ^E-regulated sHsps IbpA, IbpB and AgsA and the putative stress responsive protein STM1250 may be involved in the ability of *Salmonella* to tolerate oxidising environments. In order to address this hypothesis, *Salmonella* was grown in the presence of H_2O_2, methyl viologen (paraquat), potassium tellurite and indole, all of which have been identified as oxidising agents or inducers of a bacterial anti-oxidant response.

Previously, Testerman et al. (2002) demonstrated that Δ*rpoE* is sensitive to oxidative shock treatment of 4 mM H_2O_2 (described as high H_2O_2) but was not sensitive to 750 µM (described as low H_2O_2). In this study, an initial growth curve screen in the presence of increasing concentrations of H_2O_2 was performed and deduced that WT

SL1344 exhibited increased sensitivity to 9 mM H_2O_2 but was not severely affected by 3 mM or 6 mM H_2O_2. In comparison, the Δ*ibpAB*Δ*STM1250*Δ*agsA* was only mildly sensitive to 3 mM but increasingly more sensitive to 6 mM and 9 mM H_2O_2, with an increased lag phase. An increased lag phase has been previously shown to be characteristic of *Salmonella* treated with H_2O_2 and is understood to be due to cellular injury that prevents cell division (Watson and Schubert, 1969). But, because bacterial catalases can eventually break down excess H_2O_2 this cellular injury is not ultimately bactericidal. As a result of the initial screen, 6 mM H_2O_2 was deemed an appropriate concentration for use in sensitivity assays to determine the contribution of IbpA, IbpB, STM1250 and AgsA to oxidative stress resistance.

In order to assess the contribution of the four proteins to the Δ*ibpAB*Δ*STM1250*Δ*agsA* phenotype identified in the initial screen, the single and multiple mutants were also subjected to growth in 6 mM H_2O_2. While in the presence of 6 mM H_2O_2 the single mutants did not grow to the equivalent OD_{600} as the corresponding untreated sample, statistical analysis concluded that there was no significant difference in the lag phase growth rate. Interestingly, the Δ*ibpA* and Δ*ibpB* mutant growth rate and growth curve appeared similar to Δ*ibpAB* double mutant, this may be because both are required for complete chaperone function, as described in Chapter 3. In a similar manner, initial growth of the Δ*STM1250*Δ*agsA* and Δ*ibpAB*Δ*agsA* mutants were reduced in the presence of H_2O_2, but only the growth rate of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly reduced. These findings propose a first identification for a role of the sHsps in *Salmonella* resistance to oxidative stress and indicate the existence of functional redundancy between the two stress response operons. Functional overlap highlights an important consideration when analysing genome or proteome expression studies and the lack of data on these genes to date obtained via global mutagenesis studies.

Interestingly, exposure of *Salmonella* to the alternative oxidants (methyl viologen, potassium tellurite and indole) did not produce the same phenotype, suggesting that the sensitivity of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant is limited to H_2O_2-induced oxidative stress. One possible explanation for the response observed is that H_2O_2 exposure leads to a decrease in intracellular ATP (Osorio et al., 2003, Winter et al., 2005) and inactivation of the ATP-dependent chaperone, DnaK, has been demonstrated during H_2O_2 exposure (Winter et al., 2005). IbpAB and AgsA are ATP-independent so their presence may be crucial for protein repair under H_2O_2 oxidative stress. The precise intracellular role of STM1250 and its dependence on ATP for function is currently unclear so it is not possible to conclude whether this may also be

possible for STM1250. It is also unclear as to whether the alternative oxidants used in this study also induce a reduction in cellular ATP in *Salmonella*. *E. coli* tellurite resistant cells, carrying the *tehAB* resistance genes, do not exhibit a drastic ATP reduction on exposure to tellurite (Lohmeier-Vogel et al., 2004). Since *Salmonella* encodes the *tehAB* genes in its genome, it could be implied that its ATP levels are not affected by tellurite, but this would require further investigation. Although potassium tellurite has been reported to increase *ibpA* promoter activity, the exact contribution of IbpA to tellurite resistance is unclear. Moreover, in contrast to *E. coli*, *Salmonella* carries extra tellurite resistance genes in its genome (*STM1808* and *year*) which likely contribute to tellurite resistance in a far greater manner than the sHsps and STM1250 in this study (Johnston, 2017).

A previous study investigating the response of *Salmonella* to H_2O_2 showed that in contrast to the findings in this thesis, an Δ*ibpA* mutant was sensitive to H_2O_2 induced oxidative stress (van der Heijden et al., 2016). However, the background strain used for the construction of the mutant was the HpxF$^-$ parent strain (deficient in the three catalase genes, *katE*, *katG* and *katN* and the two alkyl hydroperoxidase genes *ahpC* and *tsaA*). Therefore, the WT was already significantly compromised in its ability to cope with oxidative stress as it lacks the core enzymes required for detoxification of H_2O_2 and its downstream ROS products (Hébrard et al., 2009, van der Heijden et al., 2016). In the same study, it was found that an influx of H_2O_2 into the cell occurs in *Salmonella ibpA* mutant and this was dependent on the OmpC porin. Both OmpC and OmpD have both been attributed with a role in H_2O_2 transport into the cell and the presence of ROS can regulate porin expression (Morales et al., 2012). It could be suggested that in this thesis, in the quadruple mutant an increased concentration of H_2O_2 enters the cell via outer membrane porins and when the IbpAB/STM1250/AgsA proteins are not present, *Salmonella* is unable to mount an appropriate response in order to cope with the intracellular oxidative stress. The individual contributions and importance of the four genes of interest may be better elucidated through the generation of single mutations in the HpxF$^-$ parent strain in future studies.

Although the results in this chapter identify novel roles for the *Salmonella ibpAB*, *STM1250* and *agsA* genes in H_2O_2 tolerance, the precise mechanisms remain unclear. Since IbpAB and AgsA are characterised as molecular chaperones, involved in preventing irreversible protein aggregation during heat stress, it can be implied that they play a similar role during oxidative stress. Their precise chaperone targets, in different stress-inducing conditions, have not been studied in detail, particularly in *Salmonella*. In *E. coli,* IbpA and IbpB are reported to protect alcohol dehydrogenase

(AdhE) from oxidative damage (Matuszewska et al., 2009) and AdhE has been reported to function as a scavenger of H_2O_2 in *E. coli* (Echave et al., 2003).

In order to reveal whether the four proteins specifically assist in preventing oxidative damage, further studies focussing on the extent of protein damage occurring in the mutants on exposure to H_2O_2, via measurement of protein carbonylation, would be beneficial. Moreover, it is not known whether the proteins in this study simply repair damaged proteins or also promote the folding and transport of bacterial catalase for the breakdown of the ROS under H_2O_2 treatment. Therefore, an investigation of the catalase activity of treated cells would also provide interesting insights.

Finally, the oxidative phenotype associated with the deletion of the genes in this study, in particular those which possess a 5' UTR RNA thermometer (*ibpA* and *agsA*), indicates their expression under conditions that are not limited to heat stress. Since H_2O_2 and its associated ROS cause DNA damage, studies should consider assessing the activity of the RNA thermometer promoter regions under stresses such as H_2O_2 treatment. Although at present the RNA secondary structures are understood to be regulated in a temperature-dependent manner, regulation of such structures by other environmental factors is possible; for example as highlighted above, *rpoS* UTR secondary structure formation and melting is controlled in a pH dependent manner (Audia et al., 2001).

4.3.3 A novel role for IbpA, IbpB, STM1250 and AgsA in intramacrophage survival

Considering the H_2O_2 and ATR phenotypes associated with the Δ*STM1250* and Δ*ibpAB*Δ*STM1250*Δ*agsA* mutants presented in this chapter and that macrophages are a site of acidification and ROS production, we hypothesised that the IbpAB, STM1250 and AgsA proteins may be important for *Salmonella* survival in the intramacrophage environment.

Following infection of IFN-γ activated macrophages, intracellular CFU/mL of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant was significantly reduced compared to WT at 2 h and 24 h post-infection. In addition, a significant reduction in survival of the quadruple mutant compared to the Δ*ibpAB* mutant further indicated functional redundancy between the *ibpA-B* and *STM1250-agsA* operons. Again, the lack of a significant phenotype for the mutants, with exception of the quadruple mutant strain, may explain

the distinct lack of data obtained and published via TraDIS studies on the roles of these genes during infection of cell lines and animal hosts.

Since the Δ*STM1250*Δ*agsA* mutant was not significantly attenuated in macrophages, it could be implied that SCV acidification is not responsible for any attenuation observed. Instead, considering the H_2O_2 sensitivity of Δ*ibpAB*Δ*STM1250*Δ*agsA*, we hypothesised that macrophage ROS production was responsible for the reduction in surviving intracellular CFU/mL of this mutant. Although macrophages release H_2O_2 in the femtomolar concentration, Amatore et al. (2006) suggested that local concentrations are extremely high and can be in the tens of millimolar range, thereby also supporting the use of the concentration of 6 mM in the earlier part of this chapter. Inhibition of the NADPH oxidase with apocynin restored the number of intracellular CFU/mL of the mutant to WT levels at both 2 h and 24 h post-infection, suggesting that the four proteins function to protect *Salmonella* against macrophage induced oxidative stress.

Although SCV acidification is not predicted to be responsible for Δ*ibpAB*Δ*STM1250*Δ*agsA* attenuation in this case, due to lack of intramacrophage phenotype for Δ*STM1250*, it would still be interesting to investigate the effects of inhibiting SCV acidification on the survival of the different mutants. From the point of view of the host, the purpose of SCV acidification is to prevent replication of *Salmonella*, but it has been reported that SCV acidification is actually required for *Salmonella* intramacrophage survival, likely due to low pH acting as an environmental cue for the expression of virulence genes e.g. SPI-2. Previously, inhibition of J774 vacuolar proton-ATPases with bafilomycin A led to a significant reduction in *Salmonella* intramacrophage survival (Rathman et al., 1996). However, later studies revealed that this is cell-line dependent and inhibition of RAW264.7 acidification (both inactivated and IFN-γ activated) did not affect *Salmonella* survival (Steele-Mortimer et al., 2000). Precise details of the exact *Salmonella* genes required for survival within acidified macrophages is certainly lacking and it can be proposed that *Salmonella* specific genes, such as *STM1250*, may play important roles in this *Salmonella* niche. Although the acid sensitive phenotype of Δ*STM1250* and lack of significant intramacrophage sensitivity of Δ*STM1250* alone do not necessarily correlate, it should be highlighted that *Salmonella* ATRs are different depending on the environment encountered. There are likely different mechanisms and different regulons induced for survival in the stomach compared to survival in the SCV, and potentially on food products too (Kenney, 2019). It could be suggested that the level of expression of *STM1250* may be associated with the pH of the local environment,

or the pH of the bacterial cytoplasm. A similar, previously published example of this occurring in *Salmonella* is STM1485, which is highly upregulated during intracellular survival (Eriksson et al., 2003) and required for translocation of SPI-2 machinery (Allam et al., 2012). Later, this was shown to be dependent on the acidification of the SCV (Allam et al., 2012). However, unlike STM1250, STM1485 was dispensable for log and stationary phase ATR survival (Allam et al., 2012).

In addition to SCV acidification and ROS production, it should be noted that the role of RNS have not been considered in this work. Since all genes of interest in this study have also been shown to be upregulated by nitric oxide (Richardson et al., 2011), future studies should investigate the sensitivity of the mutants to RNS using compounds such as the NO donor DetaNONOate. In addition to ROS, macrophages produce NO via the iNOS enzyme. Inhibition of the macrophage iNOS can be achieved with the nitric oxide synthase inhibitor L-NAME (N omega-Nitro-L-arginine methyl ester hydrochloride), which would prevent the production of intracellular RNS. Additional studies such as this may enlighten further roles of the proteins in this study or indeed confirm that the proteins specifically protect against macrophage ROS.

Although global mutagenesis studies in a range of hosts fail to directly associate the genes in this study with a role in infection, a recently published SalCom compendium (Canals et al., 2019) compared expression levels of *S.* Typhimurium 4/74 and the iNTS serovar D23580. In support of the data presented in this chapter, *STM1250* was shown to be expressed during macrophage survival, and interestingly was more highly expressed in the D23580 strain compared to 4/74. As presented in Chapter 3, *S.* Typhimurium (NTS) STM1250 is homologous to that of the iNTS strain and therefore the increased expression of *STM1250* in D23580 observed by Canals et al. (2019) should be investigated further, and the intracellular survival assays performed in this thesis should be repeated with a D23580 Δ*STM1250* mutant.

4.3.4 *STM1250* and *AgsA* – novel members of SPI-11?

Analysis of the genomic region of the *Salmonella* specific genes *STM1250* and *agsA* revealed that they are located downstream of the SPI-11 region. Due to the lower than average percentage GC content of these genes and the fact that a number of genes within SPI-11 are also regulated by σ^E, we propose that *STM1250* and *AgsA* form part of SPI-11. Genes within this island are involved in virulence and outer membrane remodelling during intracellular infection. These factors point towards involvement of STM1250 and AgsA during infection. In addition, SPI-11 members are regulated by the PhoPQ TCS. As described in Chapter 3, *STM1250* is predicted to

be PhoPQ-regulated due to the presence of a conserved PhoP binding motif located 5' of the *STM1250* start codon. qRT-PCR was used to investigate PhoP induction of *STM1250* during exposure to polymyxin B. However, expression of *STM1250* in the WT and Δ*phoP* mutant was not significantly different, suggesting that under these conditions PhoP does not induce *STM1250* expression. Further qRT-PCR studies would be valuable, since standard error was high, making it difficult to confidently draw conclusions from the data. If functional overlap is exhibited in the presence of polymyxin B as hypothesised, *STM1250* may be more highly expressed in the triple or Δ*ibpAB* double mutant, therefore qRT-PCR studies should also address its expression and potential PhoP regulation in these strains too. Considering the acid sensitivity of Δ*STM1250*, it would also be interesting to investigate *STM1250* expression in the WT and Δ*phoP* mutants in acidic conditions. Finally, it would be valuable to experimentally confirm whether *STM1250* is regulated by PhoP by electrophoretic mobility shift assay (EMSA) to investigate DNA-protein interactions.

4.3.5 Summary

In summary, in this chapter novel roles for the sHsps IbpAB and AgsA and the putative stress response protein STM1250 in protection against H_2O_2-induced oxidative stress have been reported. Furthermore, for the first time, these proteins have been associated with *Salmonella* intracellular macrophage survival and protection against NADPH oxidase derived ROS. In addition to the functionally overlapping roles between the four proteins in the presence of oxidative stress, a novel STM1250 specific role has been identified in the *Salmonella* ATR.

The results presented in this chapter provide highly interesting future avenues of research and emphasise the importance that studies on sHsps and their functional partners should not be limited to heat stress investigations.

Chapter 5 Investigating protein-protein interactions between STM1250 and the IbpA, IbpB and AgsA sHsps

5.1 Introduction

In this thesis, novel and overlapping roles have been identified for the *Salmonella* sHsps IbpA, IbpB and AgsA, and the putative stress response protein STM1250. The first half of this chapter investigates whether protein-protein interactions are responsible for this functional redundancy using the bacterial two-hybrid assay (BTH). In addition, for the first time, this thesis has shown that STM1250 is associated with the *Salmonella* ATR, a function independent of the sHsps. The second part of this chapter addresses the purification of the STM1250 protein for further biochemical study.

5.1.1 The bacterial two-hybrid assay

The BTH system was first described by Karimova et al. (1998) and allows for the detection of protein-protein interactions via heterologous expression in the adenylate cyclase deficient (*cya*$^{-}$) *E. coli* strain BTH101. Detection of interactions relies upon the adenylate cyclase protein from *Bordetella pertussis* (*B. pertussis*), which is formed of two domains T25 and T18, as a reporter of protein interactions. In the functional enzyme, adenylate cyclase is responsible for the conversion of ATP to the signalling molecule cAMP. In *E. coli*, cAMP binds to the catabolite activator protein (CAP), a transcriptional activator that controls the expression of lactose and maltose catabolising genes, for example the *lacZ* gene, encoding β-galactosidase. In the BTH system, the two domains of cAMP are carried separately on two plasmids and are non-functional. However, genes of interest can be cloned into the plasmids, so that respective proteins are fused to an individual domain of adenylate cyclase domain. When co-expressed in *E. coli*, if the proteins of interest interact the T18 and T25 domains will associate, reconstituting the functional enzyme (Figure 81). The production of cAMP, subsequent activation of CAP and expression of the *lacZ* gene allows for blue-white screening in the presence of the β-galactosidase substrate X-gal. Moreover, interactions can be quantified by β-galactosidase activity assays (Miller, 1972). The assay utilises the colourless substrate ortho-nitrophenyl-β-galactoside (ONPG) which is converted to the yellow ortho-nitrophenol (o-nitrophenol) by β-galactosidase. The amount of colour change (measured by OD_{420}) and the duration required for the colour change from colourless to yellow to occur is used to calculate Miller units, an arbitrary expression of β-galactosidase activity (Miller, 1972).

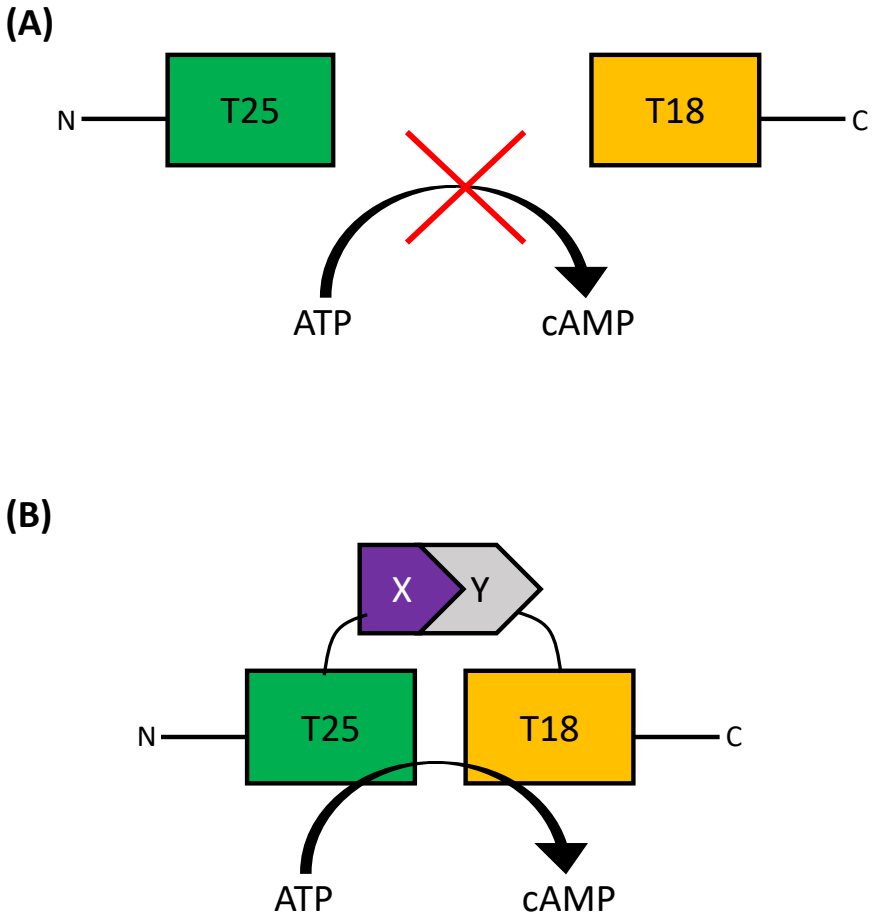


Figure 81. A schematic representation of the bacterial two hybrid (BTH) system. The BTH system utilises the adenylate cyclase gene (*cyaA*) from *Bordetella pertussis* (*B. pertussis*) which is formed of a T25 and T18 domain. Both fragments are required for the catalysis of ATP to cyclic AMP (cAMP). In the BTH system, the two domains are carried separately on two plasmids. (A) The two domains alone do not interact and will not catalyse cAMP production. (B) The domains can be fused to two proteins of interest. If these proteins interact, the two CyaA domains associate to form a functional protein, leading to the production of cAMP. Adapted from Karimova et al. (1998) and Karimova et al. (2000).

In the BTH system, four vectors are available for the fusion of the adenylate cyclase fragments to either the N- or C- terminus of the gene of interest (Figure 82). In this study, the pKT25 and pUT18C vectors were used; the CyaA fragments were fused to the N-terminus of each protein of interest. Expression of the hybrid proteins is induced by the addition of IPTG. Although IPTG is an inducer of the *lac* operon, the endogenous *lac* operon requires the cAMP/CAP complex for complete expression. Therefore, β-galactosidase activity is only observed in the presence of interacting

proteins, which result in reconstitution of the functional CyaA protein and production of cAMP (Battesti and Bouveret, 2012).

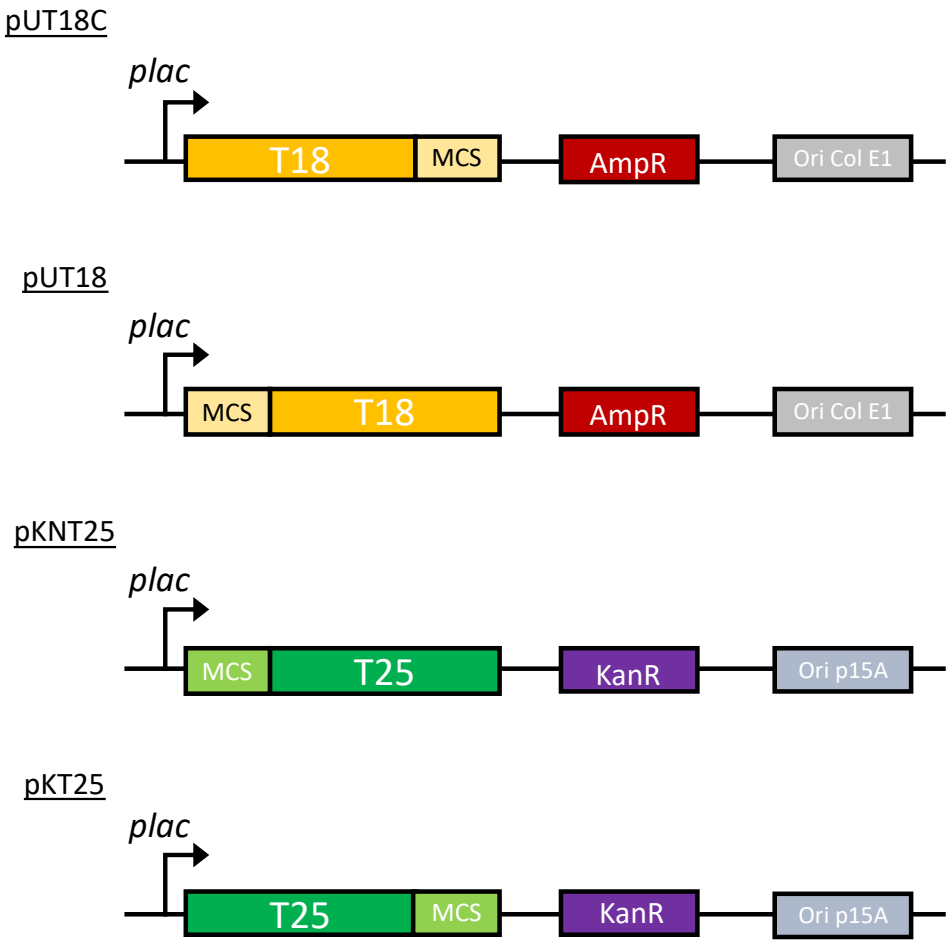


Figure 82. Bacterial two hybrid plasmids. Genes of interest can be fused with a T18 or T25 fragment of the *B. pertussis* gene at the N- or C- terminus due to the presence of a multiple cloning site (MCS) on each vector. The T18 vectors encode ampicillin resistance (AmpR) genes and the T25 vectors encode kanamycin resistance (KanR) genes, enabling co-transformation and selection in the BTH101 *cya*$^{-}$ host strain.

5.1.2 Aims

In the previous chapters, functional overlap has been identified between IbpA, IbpB, STM1250 and AgsA in the presence of polymyxin B and H_2O_2 and during intramacrophage survival. In addition, a novel role for STM1250 in the *Salmonella* ATR was reported. This chapter aims to investigate whether functional overlap exists due to protein-protein interactions to perhaps allow the formation of heteromers. Moreover, this chapter addresses the biochemical characterisation of the putative stress responsive protein STM1250.

These aims were achieved through:

- Bacterial-two hybrid assays between the four proteins of interest (IbpA, IbpB, STM1250 and AgsA).
- STM1250 protein purification trials.
- *In silico* analysis of potential STM1250 structures and intracellular localisation.

5.2 Results

5.2.1 Investigating protein-protein interactions between IbpA, IbpB, AgsA and STM1250 using the bacterial two hybrid assay

The *ibpA, ibpB, STM1250* and *agsA* genes were each cloned into the pKT25 and pUT18C vectors and co-expressed in different combinations in the *E. coli cya*$^{-}$ strain, BTH101. Expression of each protein was induced by the addition of 0.5 mM IPTG to LB agar and bacteria were maintained at 30 °C for 3 days (according to the Euromedex BACTH protocol). *E. coli* BTH101 carrying pKT25 and pUT18C empty vectors were used as a negative control. The lack of blue colour associated with the negative controls at Day 3 confirmed that any positive interactions detected (i.e. blue colonies) were due to interactions between the cloned proteins of interest, and not due to background induction of the *lac* operon or β-galactosidase activity. Moreover, the positive control vectors fused to two fragments of the leucine zipper gene (zip) were used to confirm that the assay was indeed functional. Images were taken each day and scored for positive interactions on the third day (Figure 83, Figure 84, Figure 85 and Figure 86).

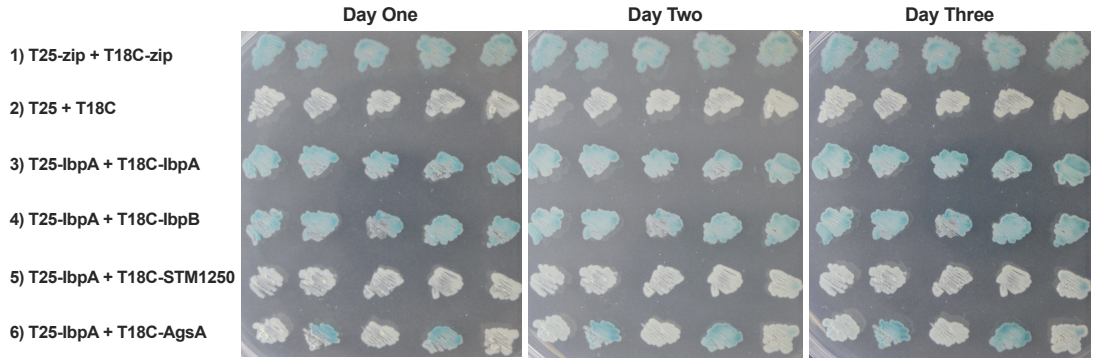


Figure 83. Bacterial two hybrid assay to test N-terminal protein-protein interactions between IbpA and IbpA, IbpB, STM1250 and AgsA. BTH101 was transformed with the following plasmid pairs 1) T25-zip + T18C-zip, 2) T25 + T18C, 3) T25-IbpA + T18C-IbpA, 4) T25-IbpA + T18C-IbpB, 5) T25-IbpA + T18C-STM1250 and 6) T25-IbpA + T18C-AgsA. Five colonies per transformant were patched onto LB agar containing 0.5 mM IPTG and 40 µg/mL X-Gal. Images were obtained after 1-, 2- and 3-days incubation at 30 °C. Blue indicates a positive interaction (as seen in positive control (1)) and white indicates no interaction (as seen in negative control (2)).

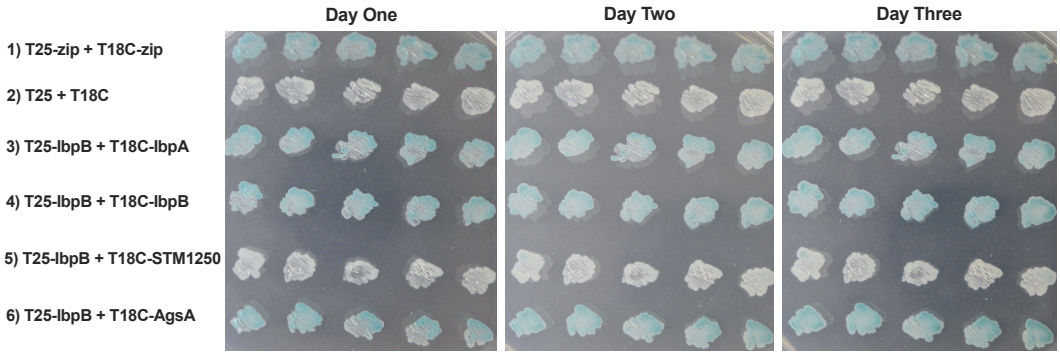


Figure 84. Bacterial two hybrid assay to test N-terminal protein-protein interactions between IbpB and IbpA, IbpB, STM1250 and AgsA. BTH101 was transformed with the following plasmid pairs 1) T25-zip + T18C-zip, 2) T25 + T18C, 3) T25-IbpB + T18C-IbpA, 4) T25-IbpB + T18C-IbpB, 5) T25-IbpB + T18C-STM1250 and 6) T25-IbpB + T18C-AgsA. Five colonies per transformant were patched onto LB agar containing 0.5 mM IPTG and 40 µg/mL X-Gal. Images were obtained after 1-, 2- and 3-days incubation at 30 °C. Blue indicates a positive interaction (as seen in positive control (1)) and white indicates no interaction (as seen in negative control (2)).

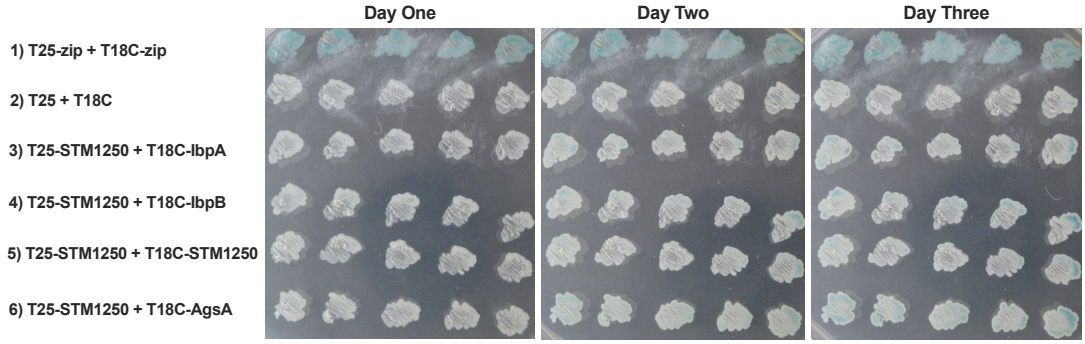


Figure 85. Bacterial two hybrid assay to test N-terminal protein-protein interactions between STM1250 and IbpA, IbpB, STM1250 and AgsA. BTH101 was transformed with the following plasmid pairs 1) T25-zip + T18C-zip, 2) T25 + T18C, 3) T25-STM1250 + T18C-IbpA, 4) T25-STM1250 + T18C-IbpB, 5) T25-STM1250 + T18C-STM1250 and 6) T25-STM1250 + T18C-AgsA. Five colonies per transformant were patched onto LB agar containing 0.5 mM IPTG and 40 µg/mL X-Gal. Images were obtained after 1-, 2- and 3-days incubation at 30 °C. Blue indicates a positive interaction (positive control (1)) and white indicates no interaction (as seen in negative control (2)).

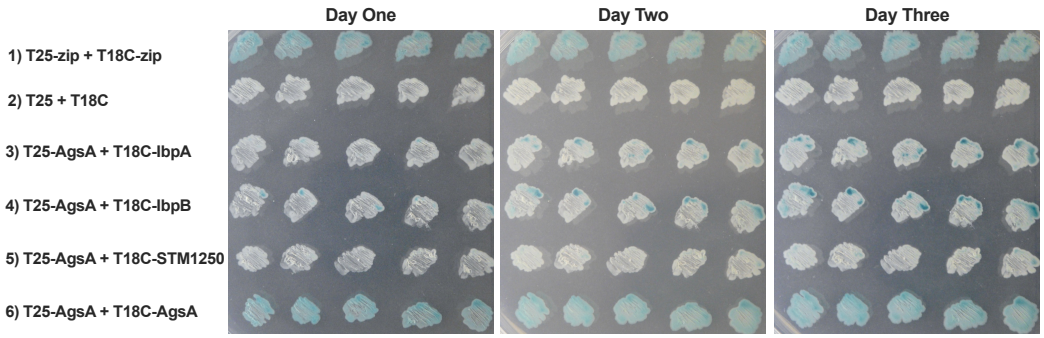


Figure 86. Bacterial two hybrid assay to test N-terminal protein-protein interactions between AgsA and IbpA, IbpB, STM1250 and AgsA. BTH101 was transformed with the following plasmid pairs 1) T25-zip + T18C-zip, 2) T25 + T18C, 3) T25-AgsA + T18C-IbpA, 4) T25-AgsA + T18C-IbpB, 5) T25-AgsA + T18C-STM1250 and 6) T25-AgsA + T18C-AgsA. Five colonies per transformant were patched onto LB agar containing 0.5 mM IPTG and 40 µg/mL X-Gal. Images were obtained after 1-, 2- and 3-days incubation at 30 °C. Blue indicates a positive interaction (positive control (1)) and white indicates no interaction (as seen in negative control (2)).

BTH assays identified positive homogeneous protein-protein interactions between the three sHsps and strong positive interactions were also observed between IbpA and IbpB. On the other hand, AgsA and IbpA only exhibited weak interactions. Interestingly, when fused to the T25 and T18 fragments respectively, IbpB and AgsA displayed interactions and all five transformants showed a blue colour on LB X-gal media after 3 days (Figure 84). However, this was not replicated in the AgsA-T25 and IbpB-T18 transformants (Figure 86) and only small indications of protein-protein interactions were visible by the third day.

In contrast to the sHsps, STM1250 displayed very weak to no interactions in the homogenous or heterogeneous transformants. After 3 days, small areas of the colonies for all interactions displayed a blue colour. Although this was not observed in the negative controls it is not possible to confidently conclude that this is solely due to protein-protein interactions.

Table 17. Summary of protein-protein interactions identified by the bacterial two hybrid assay. Genes were cloned into the pKT25 or pUT18C vectors, as indicated by the column header, and co-expressed in *E. coli*. Five transformants for each gene combination were screened on LB agar containing 40 µg/mL X-gal. The number of transformants displaying a blue colour, and therefore were deemed to be positive for protein-protein interactions was scored, providing an interaction score out of a total of five.

pKT25	pUT18C	Interaction score
ibpA	*ibpA*	5/5
ibpA	*ibpB*	5/5
ibpA	*STM1250*	0/5
ibpA	*agsA*	2/5
ibpB	*ibpA*	5/5
ibpB	*ibpB*	5/5
ibpB	*STM1250*	0/5
ibpB	*agsA*	5/5
STM1250	*ibpA*	1/5
STM1250	*ibpB*	1/5
STM1250	*STM1250*	1/5
STM1250	*agsA*	2/5
agsA	*ibpA*	2/5
agsA	*ibpB*	2/5
agsA	*STM1250*	1/5
agsA	*agsA*	5/5

5.2.2 β-galactosidase assays reveal novel protein-protein interactions between quadruple mutant proteins

In order to support the identification of protein-protein interactions by the BTH blue/white screening method, protein-protein interactions were also quantified by β-galactosidase activity assays. The *E. coli cya*$^{-}$ host strain, co-expressing the pKT25 and pUT18C constructs was also used for this purpose. Expression of each gene fused to either the T18 or T25 fragment was induced by the addition of 0.5 mM IPTG. β-galactosidase activity, and therefore protein-protein interactions, was assessed in exponentially growing cultures and expressed in Miller units (Figure 87).

β-galactosidase activity assays confirmed homogeneous interactions between all three of the sHsps and heterogenous interactions between IbpA and IbpB (T25-IbpB + T19C-IbpA), as seen previously in the BTH assays (Figure 84). Homogeneous interactions between AgsA monomers were extremely strong with a β-galactosidase activity value of 3000 Miller units. Interestingly, β-galactosidase activity assays identified that STM1250 may weakly form homogeneous interactions with itself and heterogeneous interactions with the AgsA sHsp.

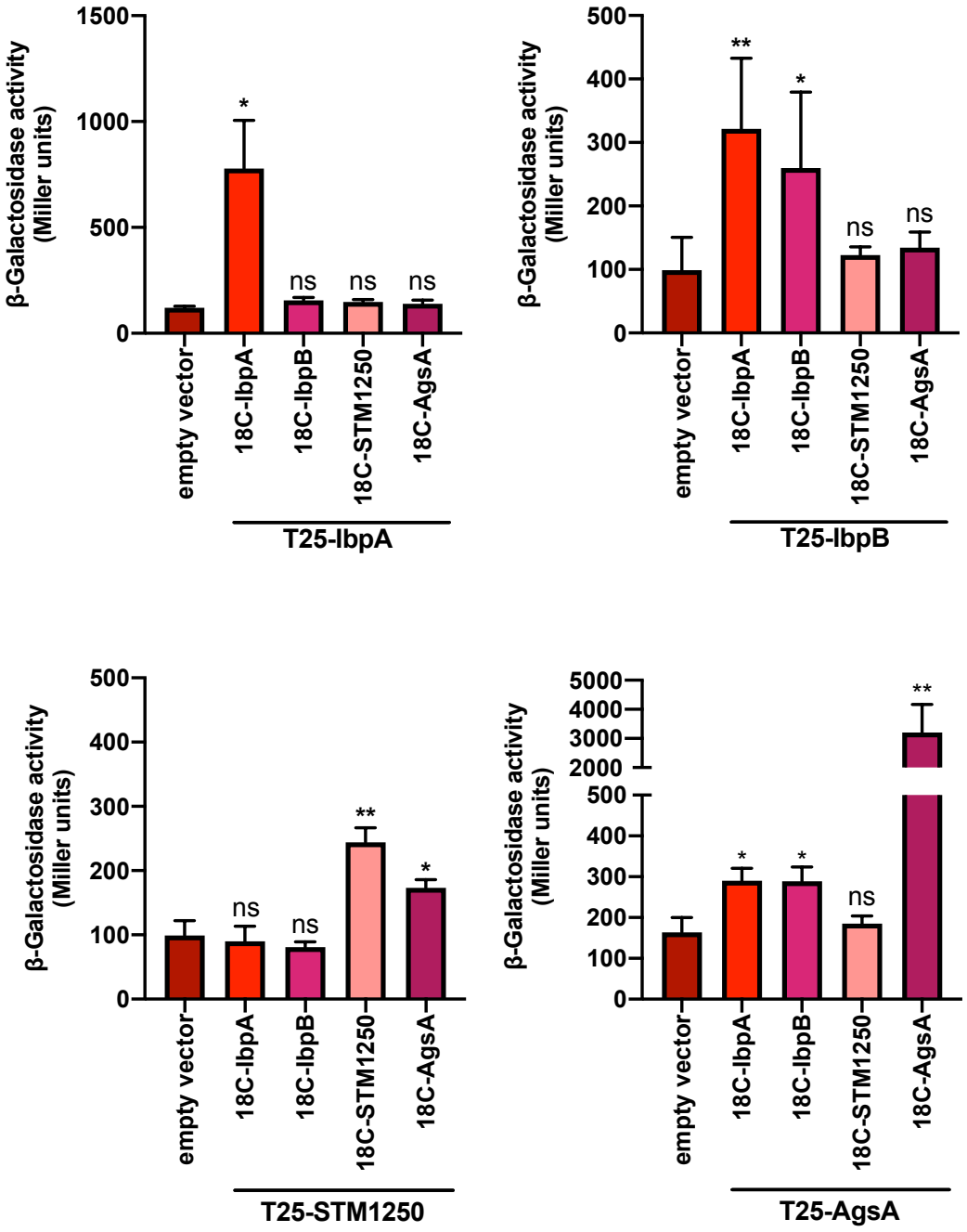


Figure 87. β-galactosidase activity assays reveal protein-protein interactions between IbpA, IbpB, STM1250 and AgsA. Genes were cloned into the pKT25 (T25) or pUT18C (18C) BTH vectors. *E. coli* BTH101 was grown to mid-exponential phase co-expressing two proteins of interest, in the presence of 0.5 mM IPTG to induce expression. Data are the means of five replicates and error bars are SEM. Protein interactions (significant β-galactosidase activity) confirmed by Student's *t*-test vs empty vector control; ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$.

The results of both the BTH and β-galactosidase activity assays are summarised in Table 18 in order to directly compare the two methods used. In most cases, where positive interactions were identified in the BTH assay this was confirmed by significantly increased β-galactosidase activity compared to empty vector controls. A notable exception was that BTH identified interactions between T25-IbpB + T18C-AgsA were not confirmed by the β-galactosidase activity assay. Coupled to the lack of interaction detected by the BTH and β-galactosidase activity assays using the T25-AgsA + T18C-IbpB constructs, this suggests that interactions between these two sHsps are either weak or transient. One further exception was that positive interactions identified in the BTH assay between T25-IbpA + T18C-IbpB were not statistically confirmed in the β-galactosidase activity assay.

In terms of negative interactions identified in the BTH assay, these were confirmed using the β-galactosidase activity assay with a lack of statistical significance. Exceptions to this included the identification of homogeneous interactions between STM1250 (T25-STM1250 + T18C-STM1250) that were statistically confirmed in the β-galactosidase activity assay but were not detected in the BTH assay. Furthermore, interactions deemed to be unclear in the BTH (particularly those between T25-AgsA and the remaining proteins of interest) were identified to be statistically significant in the β-galactosidase activity assay.

Table 18. Summary of protein-protein interactions identified by BTH and β-galactosidase activity assay. Unclear interactions indicate that it was not possible to confirm a positive interaction in the bacterial two-hybrid assay (the interaction score was < 3).

Protein pair		Positive interaction in plate-based assay?	Significant Miller assay interaction?
pKT25	pUT18C		
IbpA	IbpA	Yes	Yes (p = 0.03)
IbpA	IbpB	Yes	No (p = 0.06)
IbpA	STM1250	No	No (p = 0.08)
IbpA	AgsA	Unclear	No (p = 0.38)
IbpB	IbpA	Yes	Yes (p = 0.005)
IbpB	IbpB	Yes	Yes (p = 0.02)
IbpB	STM1250	No	No (p = 0.35)
IbpB	AgsA	Yes	No (p = 0.20)
STM1250	IbpA	No	No (p = 0.78)
STM1250	IbpB	No	No (p = 0.58)
STM1250	STM1250	No	Yes (p = 0.002)
STM1250	AgsA	Unclear	Yes (p = 0.02)
AgsA	IbpA	Unclear	Yes (p = 0.02)
AgsA	IbpB	Unclear	Yes (p = 0.03)
AgsA	STM1250	Unclear	No (p = 0.61)
AgsA	AgsA	Yes	Yes (p = 0.009)

5.2.3 STM1250 Protein Purification

Continuing on from the identification of novel protein-protein interactions, in particular those involving the novel stress response protein STM1250, the second part of this chapter describes experiments performed with the aim of purifying the STM1250 protein for further biochemical characterisation and identification of interacting *Salmonella* proteins.

5.2.3.1 STM1250 overexpression trials

Protein overexpression trials were carried out in *E. coli* Top10 and *S.* Typhimurium Δ*STM1250* background strains, at varying temperatures and with different induction time periods in order to determine optimal conditions for overexpression. In all trials, bacteria were grown at 37 °C to mid exponential phase before the addition of 0.2% *L*-arabinose for induction of the pBAD promoter. Induced cultures were incubated at 15 °C, 30 °C or 37 °C for either 4 hours or overnight. Although significant overexpression of STM1250 in both host strains was difficult to observe by SDS-PAGE analysis (Figure 88A), western blotting confirmed that STM12506xHis was present in Top10 cultures induced at 30 °C and 37 °C for 4 hours (Figure 88B, Lanes 10 and 11). In comparison, STM1250-6xHis was not detected by western blotting in *S.* Typhimurium Δ*STM1250* cells under any of the conditions tested.

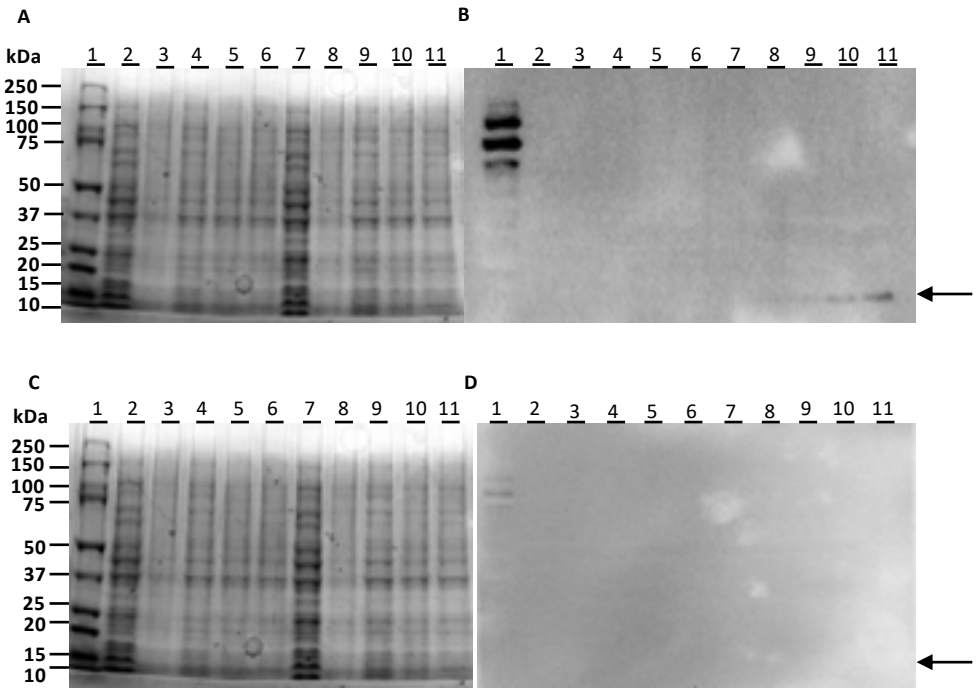


Figure 88. Small scale overexpression trials of STM12506xHis in *E. coli* WT Top10 and *S.* Typhimurium Δ*STM1250* backgrounds. *S.* Typhimurium Δ*STM1250* (A and B); *E. coli* Top10 (C and D). Bacteria were grown in 50 mL volumes to mid-exponential phase (OD_{600} 0.4) before the addition of 0.2% *L*-arabinose. Cultures were then incubated at 15 °C, 30 °C or 37 °C to induce expression of STM1250. The expected location of STM1250-6xHis (12.8 kDa) is indicated by a black arrow. In all SDS-PAGE gels and western blots the lanes are as follows: Lane 1 Precision Plus Protein Dual Colour Standards (Bioline). Lane 2 pBAD empty non-induced. Lane 3 pBAD empty induced 15 °C 4 h. Lane 4 pBAD empty induced 15 °C O/N. Lane 5 pBAD empty induced 30 °C 4 h. Lane 6 pBAD empty induced 37 °C 4 h. Lane 7 pBAD-STM1250 non-induced. Lane 8 pBAD-STM1250 induced 15 °C 4h. Lane 9 pBAD-STM1250 induced 15 °C O/N. Lane 10 pBAD-STM1250 induced 30 °C 4 h. Lane 12 pBAD-STM1250 induced 37 °C 4 h.

5.2.3.2 Large scale STM1250 expression

Based on the overexpression trial results, *E. coli* Top10 was selected as an appropriate host strain for STM1250 production and cells were induced at 37 °C for 4 hours in large scale (4x 1 L) expression cultures. STM1250 overexpression in the large-scale conditions was confirmed by SDS-PAGE (Figure 89)

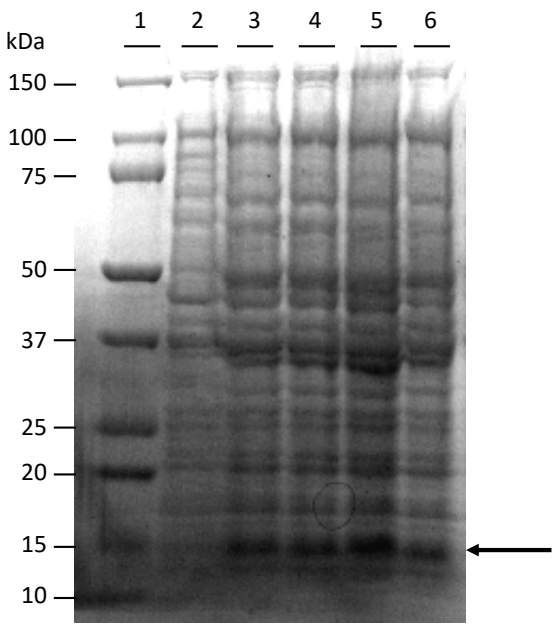


Figure 89. SDS-PAGE analysis of STM1250 overexpression trials confirmed expression of STM1250 in the *E. coli* Top10 strain. STM1250 was overexpressed in Top10 from the arabinose-inducible pBAD vector. Lane 1: Precision Plus Protein Dual Colour Standards (BioLine). Lane 2: Non-induced (0% *L*-arabinose). Lanes 3-6: Induced samples (0.2% *L*-arabinose, 37 °C for 4 hours). The presence of STM1250 is indicated by an arrow.

Protein purification was performed on an ÄKTA Pure FPLC (GE Healthcare). The elution profile and corresponding SDS-PAGE analysis of eluted fractions is shown in Figure 90. It can be observed that all fractions were significantly contaminated with multiple proteins and the initial purification did not result in the elution of pure STM1250 protein. However, the presence of the overexpressed protein could be observed in multiple fractions taken from the centre of the elution peak. Therefore, eluted fractions were pooled (Lane 2, Figure 91B) and further purified by gel filtration. Multiple elution peaks were identified and the protein content of each peak was analysed by SDS-PAGE. The presence of a pure, low molecular weight protein corresponding to that of STM1250 was confirmed (Figure 91).

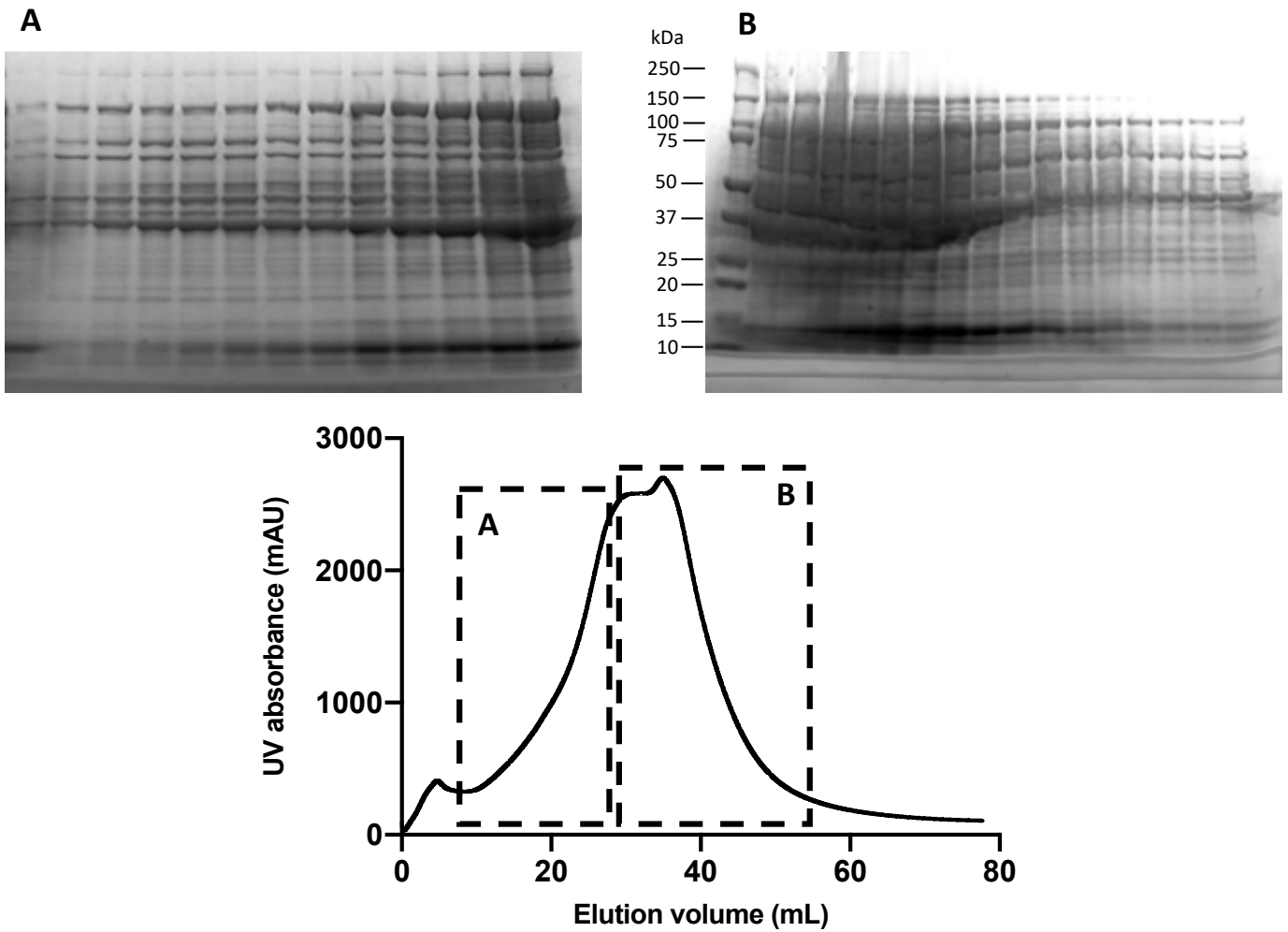


Figure 90. Elution profile and SDS-PAGE analysis of initial STM1250 purification. Alternating fractions covering the entire elution profile were analysed by SDS-PAGE. Precision Plus Protein Dual colour standards (BioRad) was used as a molecular marker.

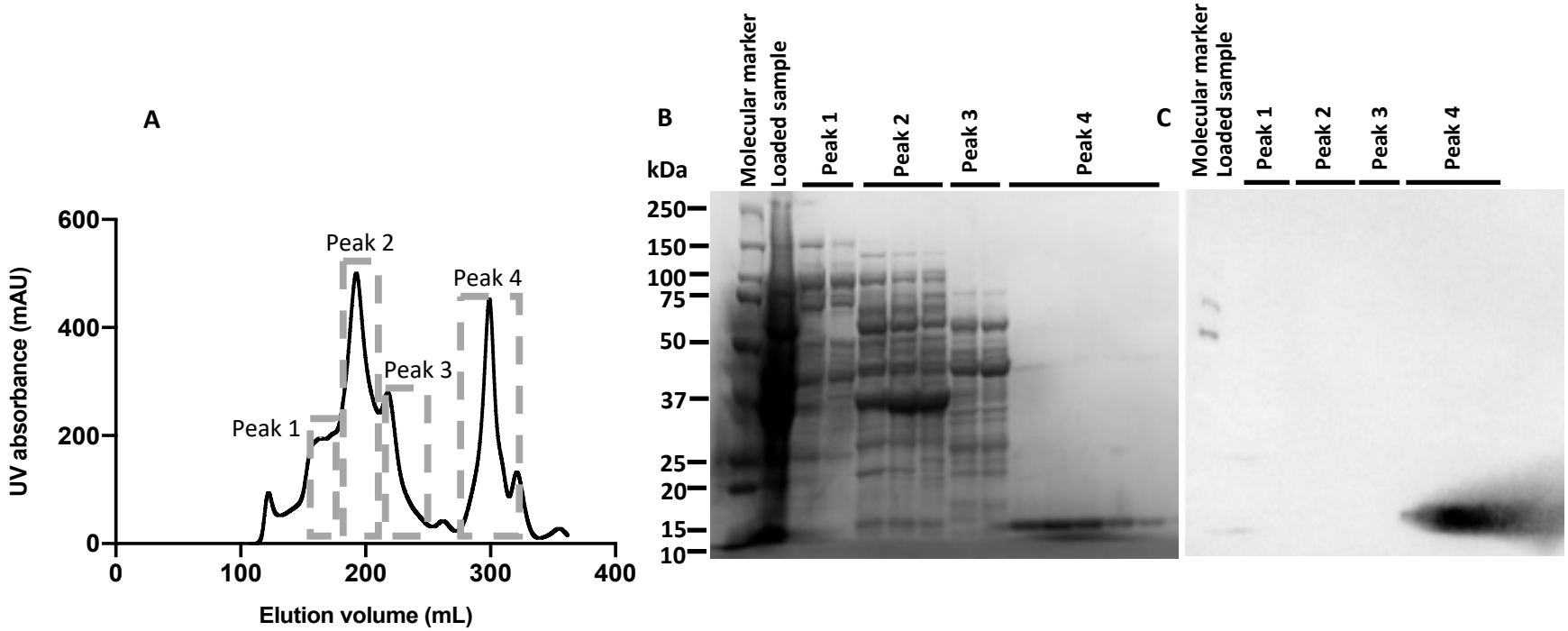


Figure 91. Elution profile and analysis after further purification of STM1250 by gel filtration. Following on from the initial purification of STM1250, peak fractions were pooled and further purified, with the resulting elution profile presented in (A). Four peaks were identified in the elution profile, as indicated by grey dashed boxes, and fractions in the centre of each peak were sampled and run on TruPAGE™ SDS-PAGE gels (Sigma) for analysis by instant blue stain and western blot, presented in (B) and (C) respectively. Peak 4 was identified to contain pure protein corresponding to the size of STM1250. Western blot analysis confirmed this to be 6xHis-tagged STM1250 (C).

5.2.3.3 STM1250 protein concentration and further study

Following gel filtration, fractions containing purified STM1250 (Peak 4, Figure 91) were pooled, concentrated and used to seed protein crystallisation trials. Crystals were obtained; however, following X-ray diffraction it was identified that the crystals isolated were in fact lysozyme crystals, not STM1250. Therefore, it was deduced that the purified STM1250 samples were contaminated with lysozyme which was used in the cell lysis protocol. Unfortunately, since the molecular weight of lysozyme is 14.3 kDa it would run extremely close to STM1250 on size exclusion chromatography columns and SDS-PAGE gels.

Large scale overexpression and protein purification was repeated in the absence of lysozyme with no further success. Future directions for this aspect of the study are discussed in more detail in the discussion at the end of this chapter and in Chapter 6.

5.2.4 STM1250 *in silico* analysis

Since the purified STM1250 obtained in this study was found to be contaminated with lysozyme, the additional planned biochemical and structural studies, including X-ray crystallography could not be performed with confidence. Therefore, *in silico* analyses were undertaken including signal peptide secretion prediction, to begin to provide insight into whether STM1250 may be secreted from the cytoplasm, in addition to homology modelling for potential structural predictions.

5.2.4.1 STM1250 is not predicted to be secreted by the Sec or Tat transportation systems

STM1250 is annotated as a putative cytoplasmic protein; however, its regulation by the extracytoplasmic sigma factor σ^E does suggest that the role of STM1250 may extend beyond the cytoplasm and it may function to maintain periplasmic/outer membrane homeostasis during stress.

The STM1250 amino acid sequence was analysed using SignalP (v5.0) (Almagro Armenteros et al., 2019), a bioinformatic tool developed for the identification of signal peptides, with the ability to differentiate between different secretion systems, and the presence of signal peptide cleavage sites.

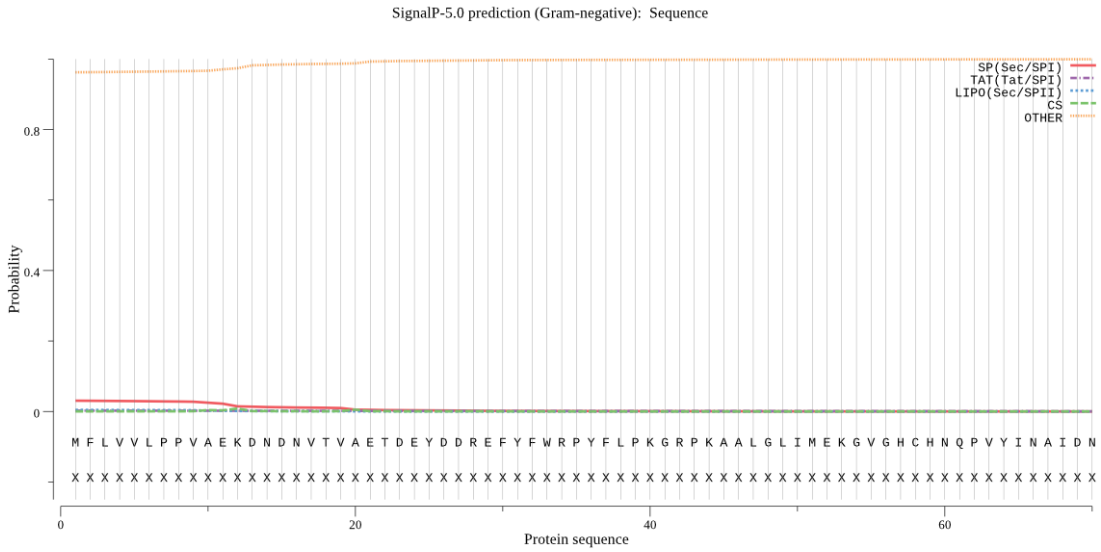


Figure 92. Signal peptide prediction indicates that STM1250 is not secreted via the Sec or Tat pathways. The STM1250 amino acid sequence was analysed by SignalP (v5.0) for the presence of signal peptides (Sec, Red line or Tat, purple dashed line) and their associated cleavage sites (CS, green dashed line). If no signal sequence or cleavage site features are detected, the sequence is described as other (orange dotted line) (Almagro Armenteros et al., 2019).

The SignalP tool did not identify the presence of a Sec or Tat signal sequence or cleavage site in the STM1250 sequence, thereby supporting the annotation of STM1250 as a cytoplasmic protein.

5.2.4.2 STM1250 protein structure homology search

STM1250 does not contain any known or conserved domains/motifs that provide any indication towards its structure or intracellular function. Since X-ray crystallography experiments were not possible in this study, *in silico* homology searching was performed. For this purpose, the online tool Phyre^2 (protein homology/analogy recognition engine v2.0) was used (Kelley et al., 2015). Phyre^2 assembles a structural model of a protein of interest, assembled from multiple alignments with protein structures available on the Protein DataBank (PDB). Unfortunately, STM1250 could not be confidently modelled to existing available structures meaning that potential structures could not accurately be determined. The modelling output is presented in Appendix E.

5.3 Discussion

In this chapter the bacterial two-hybrid assay was utilised to investigate whether protein-protein interactions exist between the *Salmonella* sHsps IbpA, IbpB and AgsA and the uncharacterised protein STM1250. Moreover, initial work was undertaken to optimise the overexpression and purification of STM1250 for future biochemical study.

5.3.1 Bacterial two-hybrid assays identify novel protein-protein interactions between STM1250 and AgsA

In Chapter 3 and Chapter 4, we demonstrated that functional overlap exists between IbpA, IbpB, AgsA and STM1250 in the presence of polymyxin B, H_2O_2-induced oxidative stress and during intramacrophage survival; specifically, the survival of the Δ*ibpAB*Δ*STM1250*Δ*agsA* was significantly reduced in all conditions compared to WT whereas the intermediate mutants (single gene deletions, double deletions (Δ*ibpAB* and Δ*STM1250*Δ*agsA*) and a triple mutant (Δ*ibpAB*Δ*agsA*) were not significantly affected. To test whether protein-protein interactions are involved in this functional overlap, and whether the proteins of interest may function in concert, a bacterial two-hybrid assay was used.

Homogeneous interactions were detected between all three sHsps. These findings were not necessarily unexpected and confirmed that the assay was appropriate for investigating protein-protein interactions, because the formation of higher order oligomers is already a described characteristic of sHsps, as highlighted in the introduction to Chapter 3 (Narberhaus, 2002). In addition to homogeneous interactions, heterogeneous interactions were also observed between the sHsps. Again, in the introduction to Chapter 3 it was highlighted that evidence of heterogeneous complexes with chaperone activity has not been detected *in vivo* but cannot be ruled out under exposure to stress-inducing conditions. In this study, BTH assays also indicated that weak interactions may exist between STM1250 monomers, potentially leading to the formation of higher oligomeric structures, and heterologous interactions may occur between STM1250 and AgsA. These interactions were confirmed by β-galactosidase assays. The identification of these novel interactions of the *Salmonella* specific proteins is extremely interesting. As indicated above, the ability to form higher order oligomers is one of the hallmarks of an α-crystallin sHsp, but stress responsive chaperones also form these structures, for example as has been shown for the chaperone/protease DegP (Jiang et al., 2008). Coupled to its

small size and the phenotypes observed thus far, current evidence suggests that STM1250 is a stress responsive protein in *Salmonella* and the protein may exert its protective/repair of stress-induced damage effects in the form of higher order oligomers. To confirm this, further study is needed via alternative methods such as pull-down assays (see below) is warranted.

Although the BTH and β-galactosidase activity assays have detected the formation of novel protein-protein interactions, it is unclear whether these are formed *in vivo* and whether they contribute to *Salmonella* physiology during stress. Since significant phenotypes for the single deletion mutants have rarely, if at all, been observed, it is unlikely that *Salmonella* relies on interactions between these proteins for their correct function.

In future work, site directed mutagenesis should be used to elucidate the specific residue(s) in each of the proteins required for protein interactions. Moreover, the individual domains of the sHsps (N-terminal region, α-crystallin and C-terminal region) could be individually fused to the T18/T25 fragments in order to identify between which domains the interactions occur. Previously, Jiao et al. (2005) reported that both the N- and C-terminal regions of IbpB are responsible for the formation of large oligomers and removal of these regions leads to the formation of dimers only, and these dimers do not possess chaperone activity. Furthermore, the requirement of the N- and C-terminal regions for IbpA oligomerization has been implicated in the phytopathogenic mycoplasma *Acholeplasma laidlawii*, of which IbpA is the only sHsp present (Chernova et al., 2020).

Extending investigations beyond the proteins in this study should also be considered. This could take both a targeted and broad whole-cell approach with pull-down assays utilising denatured *Salmonella* whole-cell lysates. For a targeted approach, the BTH assay could be utilised with specific proteins of interest. In a recent study, van der Heijden et al. (2016) suggested that IbpB interactions with the OmpC porin control the influx of H_2O_2 into the *Salmonella* intracellular space. OmpC has also been suggested to interact with IbpA in *E. coli* (Butland et al., 2005). Therefore, it would be interesting and extremely beneficial in the identification of their intracellular functions, if interactions between the sHsps, STM1250 and potential functional partners were investigated. These investigations should primarily include OmpC, which may reveal mechanistic details behind the sensitivity of the Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant to H_2O_2 (Chapter 4). Furthermore, interaction studies could be extended to cover the molecular heat shock chaperones, for example DnaK, DnaJ and GrpE. Interestingly, in *E. coli* IbpB has also been shown in a BTH to interact with NlpI (Tao et al., 2015).

NlpI is a lipoprotein which in *E. coli* is involved in interactions with host intestinal epithelial cells, immune evasion and cell division; Tao et al. (2015) demonstrated that overexpression of NlpI in *E. coli* inhibited growth and disrupted FtsZ localisation at the septum and formation of the cell division plane and this was dependent on IbpA and IbpB. Furthermore, overexpression of NlpI led to increased OM localisation of IbpA and IbpB, raising further questions to their intracellular location under different stress-inducing conditions (Tao et al., 2015). Significant upregulation of *ibpA, ibpB* and the heat shock response genes *groEL-groES*, *dnaK* and *clpB* mRNA was also observed when NlpI was overexpressed (Tao et al., 2015). It was not considered that this upregulation may be due the overexpression of the NlpI protein and resulting inclusion body formation. Interestingly, in *Salmonella* the roles for NlpI do not include cell division but it has been shown that NlpI is important in *S.* Typhimurium cold acclimatization (Rouf et al., 2011). Considering the cold shock sensitivity of the Δ*ibpAB* and Δ*ibpAB*Δ*agsA* mutants presented in Chapter 3, interactions with NlpI should be investigated.

Finally, in Chapter 4, a novel role for STM1250 in the protection of acid-adapted *Salmonella* against extreme acid shock was described. Considering the non-canonical activation of σ^E under acid stress (Muller et al., 2009), we suggested that future studies should investigate whether the σ^E stress response is fully activated in an Δ*STM1250* mutant, and these investigations may be extended to determine whether STM1250 interacts with any of the components of the regulated intramembrane proteolysis cascade of σ^E activation.

5.3.2 STM1250 protein purification

As highlighted throughout this thesis, STM1250 is poorly characterised and its intracellular role is unknown. To assist in its characterisation, in addition to the phenotypic studies reported in this thesis, it would be extremely beneficial to obtain purified protein to enable structural determination and for functional/biochemical assays to be performed.

In this thesis, we were unable to purify the STM1250 protein for further study and there are multiple stages of the purification process that require further optimisation. Although successful overexpression of STM1250 in the *E. coli* Top10 background strain was confirmed by western blot and 37 °C was selected as the optimal temperature, the level of expression was still rather poor. The Top10 strain was selected because it is unable to metabolise *L*-arabinose, therefore its concentration remains constant for induction of the pBAD promoter. Moreover, it is a recommended

host-strain for protein overexpression from the pBAD vector (Invitrogen). Despite this, it could be more beneficial to optimise the overexpression of STM1250 in *Salmonella*. Since STM1250 is specific to *Salmonella* with no conserved features, the host strain must be capable of producing and correctly folding the protein. In this study, no evidence of STM1250 overexpression in the *S.* Typhimurium Δ*STM1250* background was visible by SDS-PAGE or western blot analysis when incubated at 15 °C, 30 °C or 37 °C. Therefore, additional parameters must be investigated including the use of alternative expression vectors, inducers and inducer concentration. An additional consideration which could be explored in future studies is whether the co-expression and co-purification of STM1250 and AgsA would assist in the purification of STM1250. At present this is simply speculative but since STM1250 was identified to interact with AgsA in the BTH assays, and *STM1250* and *agsA* form an operon (Skovierova et al., 2006), co-expression and purification may increase overall yield and provide a more detailed mechanistic insight into these two *Salmonella* specific proteins.

In Chapter 5, the acid sensitive phenotype of the Δ*STM1250* mutant was rescued by the overexpression of STM1250 from the IPTG-inducible vector pET-Duet1. Therefore, this vector could be a logical starting point in future overexpression trials and since it carries two separate MCS with IPTG-inducible promoters for the expression of two different genes, it may be used to co-express STM1250 and AgsA as suggested above. In addition, vectors with cleavable epitopes should be considered so that following purification, the epitope can be removed and will not affect downstream applications; epitope removable is often preferable for X-ray crystallography as the presence of the tag can affect crystal formation.

Despite the initial low protein yield in small scale overexpression trials, SDS-PAGE analysis of the large scale (1L) grow ups identified the overexpression of a small protein running between the 10-15 kDa molecular markers, corresponding to STM1250. One-step purification did not lead to the elution of pure STM1250. In fact, as can be seen in Figure 90, all fractions were significantly contaminated with numerous proteins. In future purifications this must be addressed and different wash steps must be tested with the aim of eluting cleaner fractions in this initial single step process.

In order to remove the contaminating proteins and obtain purified STM1250, size exclusion chromatography by gel filtration was performed, using pooled fractions from the initial purification run. Gel filtration chromatography successfully separated a 10

kDa protein confirmed by western blot to contain the 6xHis-tag epitope and therefore corresponding to STM1250.

Initial structural characterisation (crystallisation trials and X-ray diffraction) identified that the STM1250 protein sample was contaminated with lysozyme protein. As detailed in Chapter 2, lysozyme is used in the lysis step of the protein purification process, prior to clearing of the cell lysate step by ultracentrifugation. Unfortunately due to this contamination, further biochemical characterisation of STM1250 could not be performed since it would be unclear if downstream results were due to the STM1250 protein or the lysozyme contaminant.

5.3.2.1 Future characterisation of STM1250

Once purified STM1250 is obtained, there are two lines of investigation that would greatly assist in the characterisation of this putative stress response protein.

Firstly, structural analyses using X-ray crystallography, analytical ultracentrifugation and native-PAGE will be extremely valuable. Structural information coupled with the phenotypes associated with Δ*STM1250* and Δ*ibpAB*Δ*STM1250*Δ*agsA* may together, enable a clearer identification of the precise role STM1250 plays in the *Salmonella* stress response.

Secondly, biochemical analyses including chaperone activity assays with model substrates (for example malate dehydrogenase and lactate dehydrogenase) will indicate whether STM1250 functions as a stress responsive chaperone, assisting in protein folding or preventing protein aggregation.

5.3.3 STM1250 *in silico* analysis

In silico signal sequence prediction identified that STM1250 is not secreted by classical secretion pathways (Sec and Tat). Despite this, non-canonical mechanisms are possible and do exist. For example, IbpA and IbpB are cytosolic sHsps, also lacking a Sec/Tat signal sequence but during cell stress have been identified to be localised at the OM (Laskowska et al., 1996, Tao et al., 2015). `For this reason, although STM1250 is annotated to be cytoplasmic, STM1250 protein localisation studies will be extremely revealing. This could be performed either by the chromosomal fusion of a detectable epitope (e.g. 6xHis) and cellular fractionation of stressed/un-stressed cells or through the use of antibodies raised specifically against STM1250. The use of antibodies may be preferred, this would minimise the risk of the addition of an epitope affecting the protein localising to its native location, especially seeing as STM1250 is such a small protein. Ultimately, localisation studies

should ensure that *Salmonella* cultures are exposed to stress (e.g. in the presence of H_2O_2 or polymyxin B) and compare protein localisation to bacteria grown in non-stressed conditions.

An accurate structural model of STM1250 with significant confidence could not be produced using the Phyre bioinformatic tool due to a lack of appropriate published models. An important consideration for the X-ray crystallographic studies suggested above is the fact that STM1250 does not contain any known or conserved domains and lacks a suitable homology model. In order to solve the phase problem and generate a structure from X-ray diffraction data, a template/model is required.

5.3.4 Summary

In this chapter, interactions between the *Salmonella* sHsps IbpA and IbpB have been confirmed. Moreover, novel interactions between the *Salmonella* specific proteins STM1250 and AgsA have been identified. These findings indicate that the functional overlap observed in previous chapters may be linked to protein interactions or co-operations between the sHsps and STM1250. This chapter also addressed the poorly characterised STM1250. Although the aims of purifying the protein could not be met, steps have begun to be made in the optimisation of the overexpression and purification process, providing the initial groundwork for future studies.

Chapter 6 General Discussion

The results of each chapter in this thesis have been discussed individually, therefore, this chapter will reiterate the major findings of the study and place the results in the wider context of *Salmonella* research. In addition, this chapter will highlight important future studies.

6.1 Context

Despite improved sanitation and health practices, *Salmonella* sp. remain a major cause of global morbidity and mortality (Majowicz et al., 2010) and one of the most common causes of zoonotic bacterial infections (European Food Safety Authority (EFSA), 2019). With worldwide cases estimated as high as 1.3 billion per year (Coburn et al., 2007), *Salmonella* infections are not only a global health concern but also a notable economic burden; for example, a recent major outbreak in the Netherlands caused by salmon contaminated with *Salmonella* Thompson was estimated to cost up to €7.5 million due to the implication of outbreak control measures and loss of productivity (Suijkerbuijk et al., 2016).

Salmonella serovars are capable of infecting humans and a range of animal hosts, causing diseases ranging from self-limiting gastroenteritis to systemic typhoidal disease. Recently, the emergence of iNTS serovars causing invasive disease is a cause for concern, particularly in sub-Saharan Africa where a rate of 34.5 cases per 100 000 has been estimated (Stanaway et al., 2019). The lack of currently available long-term vaccination for typhoidal serovars and, more notably, the lack of any licensed vaccine for NTS and iNTS serovars emphasises the pressing need for the identification of novel vaccine strains or therapeutic agents (MacLennan et al., 2014, Baliban et al., 2020). Drug-resistant outbreaks are an increasing cause for global concern with respect to many bacterial pathogens, including *Salmonella*. In 2018, a typhoid fever outbreak occurred in Pakistan caused by an extensively drug-resistant (XDR) strain of *S.* Typhi, susceptible to only azithromycin and carbapenem drugs (Klemm et al., 2018, Chatham-Stephens et al., 2019) while 90% of iNTS isolates in Malawi are classified as MDR (Gordon et al., 2008). The dependence on antibiotics for treatment of serious cases of salmonellosis, iNTS-induced bacteraemia and typhoid fever, coupled with an increasing prevalence of MDR isolates, again emphasises the pressing need for the identification of new therapeutic targets and more effective treatments. In the United States, the CDC categorises MDR *Salmonella* as a serious threat level pathogen (Brunelle et al., 2017). Furthermore, in 2018, the World Health Organisation published a list of priority pathogens to highlight particular concerning areas of increasing drug resistance. Pathogens were

categorised at a critical (carbapenem resistant *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae*), high (including fluoroquinolone resistant *Salmonella* spp. and *Campylobacter* spp.) or medium (including fluoroquinolone resistant *Shigella* spp.) need for new antibiotics (Tacconelli et al., 2018).

Salmonella stress responses contribute to *Salmonella* pathogenicity by providing resistance to antibiotics and increasing *Salmonella* survival in hostile and changing environments. During the *Salmonella* lifecycle, the bacterium is exposed to many stresses associated with the food processing industry and the host innate and adaptive immune system. These stresses include fluctuating temperatures, acidic pH, osmotic pressure, desiccation, reactive oxygen and nitrogen species, inflammation and nutrient and oxygen limitations.

6.2 The significance of the bacterial stress responses

Salmonella stress response systems function to adapt to changing and hostile environmental conditions to enable/enhance survival. The Gram-negative envelope is a crucial barrier between the intracellular and extracellular environment and therefore the ESR processes that contribute to its maintenance are essential. The stresses described above can cause serious damage to both DNA and proteins and the ESRs are vital for sensing the damage and initiating the expression of genes required for DNA or protein repair. A large majority of stress response studies published in the literature focus on the characterisation of response pathways and regulons in laboratory *E. coli* strains. Should significant advances be made in the identification of novel therapeutic targets, studies must be extended into other pathogenic bacteria such as *Salmonella*. This thesis has utilised the model NTS serovar *S.* Typhimurium and provides the basis and evidence for future work to be performed using alternative serovars such as *S.* Typhi and serovars that cause iNTS.

The σ^S general stress response, σ^H heat shock response and ESRs such as σ^E, PhoPQ and CpxAR, along with many of their regulon members, have received attention with respect to their association with *Salmonella* pathogenesis. In particular, the extracytoplasmic sigma factor σ^E is critical for *S.* Typhimurium murine infection (Humphreys et al., 1999) and is upregulated in macrophages (Eriksson et al., 2003). Due to its central role in controlling the expression of a vast regulon of genes, *Salmonella rpoE* mutants are 'super-attenuated' and are not appropriate for use as vaccine candidate strains (Rychlik and Barrow, 2005). For this reason, the regulon members, i.e. the virulence factors, stress responsive chaperones and proteases,

regulated by ESR systems may provide more appropriate alternative routes for more effective vaccine strains. With this in mind, many σ^E-regulated genes have also been implicated in *Salmonella* pathogenesis; *S.* Typhimurium *skp* chaperone mutants are attenuated in mice and *S.* Copenhagen *fkpA* mutants are attenuated for intracellular survival (Horne et al., 1997). On the other hand, *S.* Typhimurium *fkpA* mutants are not attenuated unless combined with a *surA* or *degP* deletion (Humphreys et al., 2003). This highlights two important considerations: firstly, differences in the roles and essentiality of such proteins can vary between different NTS serovars highlighting the need for extended studies, not limited to *S.* Typhimurium and *S.* Typhi. Secondly, due to their critical intracellular roles for survival during exposure to stress-inducing conditions and in pathogenesis, functional overlap can often exist between stress responsive proteins.

Identification of the σ^E regulon in *S.* Typhimurium identified 18 proteins of unknown functions, including many proteins specific to *Salmonella* (Skovierova et al., 2006). This thesis sought to determine the roles of four σ^E-regulated proteins, two of which are specific to *Salmonella* sp. Due to overlapping regulators, the formation of two operons and shared sequence homologies (detailed in Chapter 3) we hypothesised that the four genes *ibpA*, *ibpB*, *STM1250* and *agsA* functionally overlap. We sought to determine whether this was the case and furthermore, investigate the contributions of these poorly understood genes to *Salmonella* physiology and survival during stress.

6.3 Small heat shock proteins and *Salmonella* pathogenesis – roles beyond heat tolerance

As indicated above, the proteins of interest in this study included the well-conserved σ^E- and σ^H-regulated sHsps IbpA, IbpB and the *Salmonella* specific σ^E- and σ^H-regulated sHsp AgsA. Surprisingly, this group of proteins has received relatively little attention in *Salmonella*, and published studies are largely limited to heat stress or structural characterisations (Tomoyasu et al., 2003, Tomoyasu et al., 2010, Shi et al., 2011, Tomoyasu et al., 2013, Zhou et al., 2016) or heterologous overexpression in *E. coli* (Ezemaduka et al., 2018, Lv et al., 2019). To date, the only known role for these proteins in *Salmonella* is in the protection against lethal heat stress (Tomoyasu et al., 2003, Tomoyasu et al., 2010).

In this study we sought to investigate whether this group of sHsps may contribute to other aspects of *Salmonella* survival. It was hypothesised that due to their shared

regulation by σ^E, the proteins may contribute to the maintenance of envelope homeostasis, in addition to their cytoplasmic sHsp functions (under the control of the heat shock response sigma factor σ^H). In addition to the three sHsps, this thesis also focused on the hypothetical protein STM1250, which again, due to its regulation by σ^E was hypothesised to contribute to *Salmonella* stress responses and envelope homeostasis. The IbpAB and AgsA sHsps are known to share a relatively high level of amino acid sequence homology, largely due to the highly conserved α-crystallin domain present in each of the proteins (Narberhaus, 2002). STM1250 does not share this feature and is not classed as a sHsp, but Skovierova et al. (2006) identified that *STM1250* forms a σ^E-regulated operon with *agsA*. Furthermore, in the case of *STM1250*, the global regulator Fis and the TCS PhoPQ are proposed to control its expression, both of which are closely intertwined with virulence mechanisms in *Salmonella* (Monsieurs et al., 2005, Wang et al., 2013). Put together, it was hypothesised that the three sHsps, in cooperation with STM1250, may overlap in function or cooperate to increase *Salmonella* survival for pathogenesis and during exposure to extracytoplasmic stress.

In order to address potential functional overlap, in this thesis a range of mutants were created; single knockout mutants of each gene in addition to double mutants lacking either the *ibpAB* gene pair or the *STM1250-agsA* gene pair. Furthermore, a triple sHsp (Δ*ibpAB*Δ*agsA*) deletion mutant and finally a quadruple mutant lacking all four genes was generated. Initially, heat shock assays were performed in order to confirm that the response of the mutant strains generated in this study agreed with previously published data (Thomas and Baneyx, 1998, Tomoyasu et al., 2003). It was confirmed that the sHsps overlap in function in response to 50 °C heat shock and only when all three sHsps are deleted was a phenotype observed. We concluded that STM1250 does not share this overlapping role since there was no significant difference between survival of the triple sHsp mutant and the quadruple mutant following 50 °C heat shock.

Analysis of previously published RNA-seq data identified that the genes in this study are upregulated under numerous other stress-inducing conditions (indicating that their functions may not be limited to heat shock) including osmotic stress, acid shock and the SCV (Eriksson et al., 2003, Hautefort et al., 2008, Kröger et al., 2013, Cao et al., 2019, Canals et al., 2019). In *E. coli*, IbpA and IbpB have been directly linked to the protection against copper-induced oxidative stress but it is unclear whether this role is conserved in *Salmonella* (Matuszewska et al., 2008). These previous observations support the hypotheses in this thesis that the roles of IbpAB and AgsA

extend beyond that of heat shock survival and the data also suggest that *STM1250* is upregulated in similar conditions to the sHsps.

Through a broad phenotypic screen we sought to identify whether the quadruple knockout strain, or its intermediate mutants, were significantly affected by different stress-inducing conditions including salt stress, increased metal (copper and zinc) concentrations, iron depletion and antimicrobials including vancomycin and polymyxin B. Conditions were selected due to their detrimental effects on the OM and the cell envelope, in addition to the existence of studies indicating significant upregulation of any of the genes of interest under a particular stress-inducing conditions. This enabled an investigation into the contribution of IbpAB, STM1250 and AgsA to stress resistance and the maintenance of envelope homeostasis.

Across the majority of the conditions tested, there were no significant effects observed in the deletion mutant strains. An interesting observation was the increased sensitivity of the quadruple mutant to polymyxin B but a lack of sensitivity for the triple, double or single deletion mutants. This was the first indication that the four proteins of interest in this thesis overlap in function in response to an environmental stress. Since polymyxin B resistance is attributed to the ability of *Salmonella* to modify its LPS structures, follow-up experiments assessed the composition of LPS molecules extracted from WT or Δ*ibpAB*Δ*STM1250*Δ*agsA* mutant cells. Unfortunately, the results here were inconclusive and as detailed in Chapter 3, further studies are required to assess, in more detail, the composition of the LPS molecules in the mutant strains.

6.3.1 The contribution of IbpAB, STM1250 and AgsA to survival against host-associated stresses

In its role as an extracytoplasmic sigma factor, σ^E upregulates genes required for survival in the presence of many extracytoplasmic stresses. Conditions that critically require σ^E (*rpoE*) are oxidative stress survival (Testerman et al., 2002, Li et al., 2015), acid tolerance (Muller et al., 2009), intramacrophage survival and murine infection (Humphreys et al., 1999). To this end, phenotypic analyses were performed in the presence of oxidants (H_2O_2, potassium tellurite, paraquat and indole) and intramacrophage survival assays were conducted. Furthermore, the ATR of WT and mutant *Salmonella* was investigated.

Growth curves conducted in the presence of H_2O_2 identified that growth of the quadruple mutant was significantly reduced in comparison to the WT. Interestingly, this phenotype was not observed for the triple, double or single mutants suggesting

that, in a similar manner to the polymyxin B assays, functional redundancy exists between the four proteins in the presence of H_2O_2. The contribution of the *Salmonella* sHsps or *STM1250* to oxidative stress resistance have not been investigated in detail prior to this thesis. In *E. coli*, *ibpAB* mutants are not sensitive to H_2O_2 oxidative stress but overexpression of the pair of sHsps significantly increases WT survival (Kitagawa et al., 2000). We did not investigate whether overexpression of any of the proteins of interest increase *Salmonella* resistance to H_2O_2 and this is a future avenue of research that may further support the data presented in this thesis. Additional assays performed with alternative oxidants did not show the same phenotype, indicating that the contributions of IbpAB, STM1250 and AgsA to oxidative stress resistance are specific to conditions associated with H_2O_2. The possible reasons behind this were discussed in detail in Chapter 4.

Considering the ROS sensitivity of the quadruple mutant, it was hypothesised that the IbpAB, STM1250 and AgsA proteins may contribute to *Salmonella* survival within the oxidising environment of the SCV. Macrophage infection assays demonstrated that the quadruple mutant was significantly attenuated in IFN-γ activated macrophages but this was not observed in inactivated macrophages. The link between oxidative stress sensitivity and intramacrophage survival was confirmed via inhibition of the macrophage NADPH oxidase with apocynin. Previous studies have confirmed that apocynin does not affect the phagocytic activity of macrophages, nor can it function as a scavenger for ROS (Stolk et al., 1994, Vejražka et al., 2005), therefore attributing the recovery of Δ*ibpAB*Δ*STM1250*Δ*agsA* survival to the reduced generation of ROS in the SCV. As indicated in Chapter 4, this study has not addressed whether IbpAB, STM1250 or AgsA contribute to the defence against RNS, which are also produced in the SCV in the form of NO. Future studies should employ the use of NO donors, such as DETA NONOate *in vitro*, to investigate sensitivity to NO. Furthermore, inhibition of the iNOS component of the macrophage may reveal further contributions of the proteins of interest to intracellular survival.

A major hypothesis of this thesis was that IbpAB, STM1250 and AgsA contribute to the maintenance of envelope homeostasis due to their regulation by σ^E. Although their contribution to processes at the envelope have been indicated due to the sensitivity of the quadruple mutant to polymyxin B, further studies are required to confirm their precise roles. Cellular localisation studies under stress (e.g. oxidative) will be extremely valuable. The fact that the three sHsps function in an ATP-independent manner, in addition to published studies identifying IbpAB at the OM, correlates with their proposed periplasmic/OM localisation (Laskowska et al., 1996,

Tomoyasu et al., 2003, Matuszewska et al., 2008, Tao et al., 2015). Despite their identification in cellular OM fractions (Laskowska et al., 1996), how these proteins traverse the IM without a Sec/Tat signal sequence remains undetermined.

In terms of STM1250, its lack of attention prior to this study means that there is a distinct lack of information on its intracellular location, however the identification of its contribution to the ATR provides the initial work required for this to be investigated further. Hydrochloric and lactic acid have been demonstrated to cause permeabilization of the OM and release of LPS into growth media (Alakomi et al., 2000) providing further routes for investigation that STM1250 is involved in maintenance of OM integrity, under certain stress-inducing conditions. Moreover, exposure to low pH can lead to oxidative stress (Rychlik and Barrow, 2005), thereby providing a potential link between the acid and oxidative stress phenotypes presented in this thesis. However, the lack of contribution of *ibpAB* or *agsA* to survival in acidic conditions suggests that oxidative stress may not be the major killing mechanism of *Salmonella* in the acidic conditions utilised in this study.

6.4 The role of protein-protein interactions in the functional overlap between IbpAB, STM1250 and AgsA

Although overlapping roles have been assigned to the sHsps and STM1250, it remains unclear whether this is simply due to similar intracellular functions of the four proteins or whether specific interactions occur. To investigate this, the BTH and β-galactosidase activity assays were employed. The results identified that the sHsps form homogenous complexes, a typical feature of α-crystallin domain sHsps (Narberhaus, 2002). Novel homogenous interactions were also identified for STM1250, although these were very weak and will require further investigation, aided by the purification of STM1250 protein. Limited heterologous interactions were also identified. Interestingly, STM1250 and AgsA may form weakly interacting complexes and in a similar manner, AgsA may weakly interact with IbpA and IbpB. Since the BTH and β-galactosidase activity assays were performed using the *E. coli* BTH101 strain (Karimova et al., 1998, Karimova et al., 2000), the interactions observed may not be a true representation of those that occur *in vivo* in *Salmonella*. Nonetheless, the results provide an interesting starting point for further investigation, once purified proteins can be obtained. There is a lack of structural information deposited on the Protein Data Bank (PDB) for the IbpAB and AgsA sHsps. The α-crystallin domain, C-

terminal and N-terminal truncated constructs are available for AgsA (Mani et al., 2016) with resolutions of 2 angstrom (Å), 4.1 Å and 7.5 Å, respectively. As a result, all aspects of this thesis would benefit from more in-depth structural characterisation of the sHsps in addition to STM1250.

6.5 The novel stress responsive protein STM1250

STM1250 is arguably the most interesting protein in this study and prior to this thesis was entirely uncharacterised with no known intracellular role. The data presented in this thesis has identified novel phenotypes associated with a Δ*STM1250* mutant, but to advance our understanding of this protein further, purification of STM1250 is required for more in-depth biochemical study.

Initial progress was made in the purification of STM1250 however as detailed in Chapter 5, further optimisation is needed. Future studies must address the poor overexpression of STM1250 in both SL1344 and *E. coli* Top10 with the aim of identifying optimal conditions for its expression. Considering that the only phenotype associated with the Δ*STM1250* single deletion mutant in this study was a reduced ability to mount the ATR, protein overexpression may prove to be more successful in acidic conditions.

With no known conserved domains or motifs and a lack of biochemical data, it remains challenging at this stage to suggest the exact intracellular function of STM1250 based solely on phenotypic data. As a result, considering the need for new therapeutic targets it cannot be suggested based on current data whether the inhibition of STM1250, and exactly how this may be achieved, could lead to *Salmonella* attenuation.

6.6 Study limitations

Limitations of this work include the lack of phenotypic complementation with a low-copy non-inducible vector, under the control of a native promoter. Although the significant phenotypes observed in this thesis have been complemented with overexpression of STM1250, or AgsA, native complementation would allow for conclusions on the contributions of the genes of interest to survival of *Salmonella* under different stress-inducing conditions to be drawn with more confidence.

A further limitation of this thesis is that during construction of the deletion mutants, in particular the single mutant strains, the potential for transcriptional polarity to occur

was not considered. Therefore, as a result, deletion of *ibpA* or *STM1250* may have affected expression of *ibpB* or *agsA*, respectively. However, due to the additional construction of double, triple and quadruple mutants, it can be concluded that the functionally redundant phenotypes observed were specifically because of deletion of specific genes of interest and not due to transcriptional polarity. For example, during heat shock assays *ibpA, ibpB* and *agsA* single mutants were unaffected by 50 ºC heat shock, but survival of the Δ*ibpAB*Δ*agsA* mutant was reduced. Furthermore, only the Δ*STM1250* mutant was sensitive to acid shock, whilst the Δ*agsA* mutant was unaffected, and the Δ*STM1250*Δ*agsA* responded similarly to the Δ*STM1250* strain. These results indicate that the *STM1250* mutant phenotype was not due to transcriptional polarity.

6.7 *Salmonella* and food security

Due to the pressing need for novel therapeutic agents, this thesis has primarily focussed on the contribution of stress response proteins to pathogenesis. However, *Salmonella* is a zoonotic pathogen and therefore is a major problem in the food chain. Potential contributions of the IbpA, IbpB, STM1250 and AgsA proteins to survival in different animal hosts have been acknowledged in this thesis. For example, the heat shock survival roles of the sHsps may enhance *Salmonella* survival in poultry, where body temperature is 42 °C (Dawoud et al., 2017). Furthermore, it was indicated in the Introduction to Chapter 3, that an *agsA* TraDIS mutant has been shown to be attenuated in cattle (Chaudhuri et al., 2013). Heat treatment of certain foods may adapt bacteria to hostile environments, for example beef-associated *E. coli* resistant to temperatures up to 70 °C have previously been isolated (Dlusskaya et al., 2011). Since the experiments in this thesis have only been performed on *S.* Typhimurium, it would greatly benefit this field of research if studies were extended into different serovars and different infection models/hosts. Of primary interest would be *S.* Enteritidis, because alongside *S.* Typhimurium it is the most common source of foodborne salmonellosis (Velge et al., 2005). In addition, more recent outbreaks particularly in the U.S. have been caused by the *S.* Newport serovar, and outbreaks associated with the consumption of fresh produce such as tomatoes and cucumbers have occurred (Angelo et al., 2015, Carstens et al., 2019).

The identification of STM1250 as a protein involved in the ATR not only has implications in the consideration of pathogenesis but also in survival against environmental stresses associated within the food industry. Unlike the observations made in the presence of polymyxin B, H_2O_2 and during intramacrophage survival, the

contribution of STM1250 to the ATR was not a functionally redundant role shared with the IbpAB/AgsA sHsps; the sensitivity of the *STM1250* mutant was not exacerbated by additive deletion of *ibpAB* and *agsA* in the quadruple mutant.

In addition to temperature, chlorine (largely in the U.S.) and acids are routinely used in the food industry as a means for decontamination and preservation, especially in poultry products. The use of chlorine washes in the food processing industry is becoming an increasingly debated topic with concerns that it may cross-react with organic matter to form carcinogenic compounds (Lu and Wu, 2012). Therefore, research into alternative methods of decontamination has begun to increase in recent years. Both acetic acid and lactic acid have been shown to be successful in the decontamination process (Huffman, 2002). The use of acid in the food preservation industry has important implications for pathogens with sophisticated adaptive ATR such as *Salmonella*. Exposure to sublethal pH in food processing environments has the potential to induce the ATR, subsequently enhancing the ability of *Salmonella* to survive in the stomach and within the acidic SCV (Álvarez-Ordóñez et al., 2012).

The ATR-associated phenotype of the *S.* Typhimurium Δ*STM1250* mutant in this thesis, discussed above and in detail in Chapter 4, emphasises that our current understanding of the abilities of *Salmonella* to withstand this stress are not complete. Should *Salmonella* contamination of food products be avoided, an improved understanding of the underlying mechanisms of resistance and the ATR are certainly required.

6.8 Final conclusions

This thesis has emphasised that stress responsive proteins play important roles across many aspects of *Salmonella* survival in changing conditions. Furthermore, we have raised the concept of functional overlap and emphasised that if research is limited to the study of single deletion mutants this may prevent the the intracellular role of proteins from being identified, if the presence of other functional/overlapping partners can compensate for their loss.

The results in this thesis advance our understanding of the roles of four poorly characterised σ^E-regulated proteins in *Salmonella* and have identified for the first time that different levels of functional overlap exist between IbpA, IbpB, STM1250 and AgsA under certain stress conditions, particularly those associated with pathogenesis (cationic AMPs, oxidative stress and the SCV). To add to this, we have identified a novel role for the hypothetical protein STM1250, which this thesis has shown

functions as a stress-responsive protein in *S*. Typhimurium and contributes to the ATR.

In conclusion, this study contributes to our knowledge of stress response proteins in *Salmonella* and multiple avenues of further investigations have been suggested. Put together, an improved understanding of the stress-responsive mechanisms employed by *Salmonella* in pathogenesis and exposure to hostile conditions may aid in the identification of much needed novel therapeutic targets.

Chapter 7 References

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Chapter 8 Appendices

Appendix A. Media recipes.

Table A1. Media compositions and instructions for preparation.

Media Name	Recipe	Description
Luria-Bertani (LB) broth	10 g Sodium chloride 10 g Tryptone 5 g Yeast extract	Dissolve components in 1 L dH_2O and autoclave to sterilise.
Luria-Bertaini (LB) agar	10 g Sodium chloride 10 g Tryptone 5 g Yeast extract 1.5% (w/v) agar	Dissolve components in 1 L dH_2O, distribute into 200 mL volumes, add 3 g agar per flask and autoclave to sterilise.
LB top agar	10 g Sodium chloride 10 g Tryptone 5 g Yeast extract 0.75 % (w/v) agarose	Prepare sterile LB broth in 50 mL aliquots. Add 0.375 g agarose per 50 mL flask and microwave to dissolve.
Lennox broth	5 g Sodium chloride 10 g Tryptone 5 g Yeast extract	Dissolve components in 1 L dH_2O and autoclave to sterilise.

M9 salts (10X) stock	64 g sodium phosphate dibasic heptahydrate 15 g monopotassium phosphate 2.5 g sodium chloride 5 g ammonium chloride	Dissolve components in 1 L dH_2O and autoclave to sterilise.
M9 minimal media (1X)	1X M9 salts 2 mL $MgSO_4$ (1M) 20 mL glucose (20% (w/v)) 0.1 mL calcium chloride (1M) 1 mL histidine (400 mg/mL)	Filter sterilise components and add to 1L 1X M9 salts.
NCE minimal media	3.94 g KH_2PO_4 6.46 g K_2HPO_4 3.5g $NaNH_4HPO_4$ 1 mM MgSO4 0.4% (w/v) glucose 400 µg/mL histidine	Dissolve components in 1 L dH2O and autoclave to sterilise. Add 0.4% (w/v) glucose (filter sterilised) and 400 µg/mL histidine (filter sterilised) after autoclaving.

UCB indicator agar (Green agar)	8 g tryptone 1 g yeast extract 5 g sodium chloride 1.5% (w/v) agar Additions per 190 mL 4 mL glucose (40% (w/v)) 5 mL alizarin yellow (2.5% (w/v)) 650 µL aniline blue (2% (w/v))	Dissolve components in 950 mL dH_2O. Distribute into 190 mL aliquots in 200 mL flasks and add 3 g agar per flask. Autoclave to sterilise. Add additions after autoclaving and cooling to ~ 50 °C.

Appendix B. Buffers and solutions

Table B1. Buffer compositions and instructions for preparation.

Buffer	Recipe	Description
0.1 M phosphate buffer	0.06 M Na_2HPO_4 x $7H_2O$ 0.04 M NaH_2PO_4 x H_2O	Dissolve in dH_2O and adjust to pH 7.0.
Z-buffer	0.06 M Na_2HPO_4 x $7H_2O$ 0.04 M NaH_2PO_4 x H_2O 0.01 M KCl 0.001 M $MgSO_4$ 0.05 M β-mercaptoethanol	Dissolve in dH2O and adjust to pH 7.0. Can be stored at 4 °C without β-mercaptoethanol. Add β-mercaptoethanol directly before use.
Infection assay lysis buffer	1% (v/v) triton X-100 0.1% (w/v) SDS	Dissolve in 1X PBS
LPS extraction lysis buffer	1 M Tris pH 6.8 2% (w/v) SDS 4% (v/v) β-mercaptoethanol	

TBE (10X)	1 M Tris base 1 M Boric acid 20 mM EDTA	Dissolve components in 1 L dH_2O
6xHis loading buffer	50 mM Tris HCl pH 7.2 250 mM NaCl	Dissolve components in dH2O and filter sterilise.
6xHis wash buffer	50 mM Tris HCl pH 7.2 250 mM NaCl 20 mM imidazole	Dissolve components in dH2O and filter sterilise.
6xHis elution buffer	50 mM Tris HCl pH 7.2 250 mM NaCl 250 mM imidazole	Dissolve components in dH2O and filter sterilise.
6xHis gel filtration buffer	50 mM Tris HCl pH 7.2 100 mM NaCl	Dissolve components in dH2O and filter sterilise.

Appendix C. Restriction enzymes used in this study.

Table C1. Restriction enzymes used in this thesis and their corresponding recognition sites. Cut sites are indicated by an arrow (↓).

Restriction enzyme	Recognition sequence
BamHI	5'…G↓GATCC…3' 3'…CCTAG↓G…5'
EcoRI	5'…G↓AATTC…3' 3'…CTTAA↓G…5'
NcoI	5'…C↓CATGG…3' 3'…GGTAC↓C…5'
XbaI	5'…T↓CTAGA…3' 3'…AGATC↓T…5'

Appendix D. Amino acid alignments between IbpA, IbpB, STM1250 and AgsA

```
IbpA         1 MRNFDLSPLYRSAIGFDRLFNLLENN-QSQSNGGYPPYNVELVDENHYRI      49
               |||:|||||.|..||||:|.|.|:|: :|||   :||||:|..|:|||||
IbpB         1 MRNYDLSPLLRQWIGFDKLANALQNSGESQS---FPPYNIEKSDDNHYRI      47

IbpA        50 AIAVAGFAESELEITAQDNLLVVKGAHADEQKERTYLYQGIAERNFERKF      99
               .:|:|||.:.:|:|..:...|.|||.....:.|..:|:||:..:.|...|
IbpB        48 TLALAGFRQEDLDIQLEGTRLTVKGTPEQPENEPKWLHQGLVMQPFSLSF      97

IbpA       100 QLAENIHVRGANLVNGLLYIELERVIPEANKPRRIEIN-------    137
               .||||:.|.||...||||:|:|.|..||...|:||.||
IbpB        98 TLAENMEVSGATFTNGLLHIDLTRNEPETIAPQRIAINERSALNS    142
```

Figure D93. Pairwise alignment of IbpA and IbpB. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

```
IbpA          1 MRNFDLSPLYRSAIGFDRLFNLLENNQSQSNGGYPPYNVELVDENHYRIA      50
                                                              ...
STM1250       1 ----------------------------------------------MFL       3

IbpA         51 IAVAGFAESELEITAQDNLLVVKGAHADEQKERTYLYQGIAERNFERKFQ     100
                :.:...||.:     .||:.|   |..||..:|.:.        |.|.:.
STM1250       4 VVLPPVAEKD-----NDNVTV---AETDEYDDREFY--------FWRPYF      37

IbpA        101 LAENIHVRGANLVNGLLYIELERVIPEA-NKPRRIEIN------------    137
                |.:........|:       :|:.:... |:|  :.||
STM1250      38 LPKGRPKAALGLI-------MEKGVGHCHNQP--VYINAIDNHYQFGWWR     78

IbpA        138 ------------    137

STM1250      79 KIVKMKGQRKWQ     90
```

Figure D94. Pairwise alignment of IbpA and STM1250. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

```
IbpA              1 --MRNFDLSPLYRSAIGFDRLFNLLENNQSQSNG-----GYPPYNVELVD     43
                    :|.....|::..::..|| ||.::...||..|     ..|.|:::..|
AgsA              1 MALRTLSALPVFADSLFSDR-FNRIDRLFSQLTGDTPVAATPAYDLQKRD     49

IbpA             44 ENHYRIAIAVAGFAESELEITAQDNLLVVKGAHADE--QKERTYLYQGIA     91
                    .|:|.:.::|.|:.|.||||......|.:.|.|.:|  :.:..::|:||.
AgsA             50 ANNYLLTVSVPGWKEEELEIETVGGNLNITGKHTEETVEDQTHWIYRGIR     99

IbpA             92 ERNFERKFQLAENIHVRGANLVNGLLYIELERVIPEANKPRRIEIN----    137
                    :.:|:..|.|.|:..|..|.|..|||.:|:.:.|||:.||::|.|.
AgsA            100 KADFQLSFSLPEHAKVNNAKLEQGLLLVEIYQEIPESEKPKKIAIESKPK    149

IbpA            138 ------    137

AgsA            150 AIEHKS    155
```

Figure D95. Pairwise alignment of IbpA and AgsA. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

```
IbpB              1 --MRNYDLSPLLR------QWIGFDKLANALQNSGESQSFPPYNIEKSDD     42
                      :|.....|:..      ::...|:|.:.|.......:.|.|:::|.|.
AgsA              1 MALRTLSALPVFADSLFSDRFNRIDRLFSQLTGDTPVAATPAYDLQKRDA     50

IbpB             43 NHYRITLALAGFRQEDLDIQLEGTRLTVKG--TPEQPENEPKWLHQGLVM     90
                    |:|.:|:::.|:::|:|:|:..|..|.:.|  |.|..|::..|:::|:..
AgsA             51 NNYLLTVSVPGWKEEELEIETVGGNLNITGKHTEETVEDQTHWIYRGIRK    100

IbpB             91 QPFSLSFTLAENMEVSGATFTNGLLHIDLTRNEPETIAPQRIAINER-SA    139
                    ..|.|||:|.|:.:|:.|....|||.:::.:..||:..|::|||..: .|
AgsA            101 ADFQLSFSLPEHAKVNNAKLEQGLLLVEIYQEIPESEKPKKIAIESKPKA    150

IbpB            140 LNS--    142
                    :..
AgsA            151 IEHKS    155
```

Figure D96. Pairwise alignment of IbpB and AgsA. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

```
IbpB              1 ---------------------------------MRNYDLSPLLRQWIGFDK     18
                                                      .|.|.|..      |..|
STM1250           1 MFLVVLPPVAEKDNDNVTVAETDEYDDREFYFWRPYFLPK------GRPK     44

IbpB             19 LANAL---QNSGESQSFPPYNIEKSDDNHY-----RITLALAGFRQEDLD     60
                    .|..|   :..|...:.|.|  ..:.||||     |..:.:.|.|:..
STM1250          45 AALGLIMEKGVGHCHNQPVY--INAIDNHYQFGWWRKIVKMKGQRKWQ--     90

IbpB             61 IQLEGTRLTVKGTPEQPENEPKWLHQGLVMQPFSLSFTLAENMEVSGATF    110

STM1250          91 --------------------------------------------------     90

IbpB            111 TNGLLHIDLTRNEPETIAPQRIAINERSALNS    142

STM1250          91 --------------------------------     90
```

Figure D97. Pairwise alignment of IbpB and AgsA. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

```
STM1250           1 --------------------------------------------------      0

AgsA              1 MALRTLSALPVFADSLFSDRFNRIDRLFSQLTGDTPVAATPAYDLQKRDA     50

STM1250           1 ---MFLVVLPPVAEKD--------NDNVTVAETDE-YDDREFYFWR----     34
                       :..|.:|...|::        |.|:|...|:| .:|:..:.:|
AgsA             51 NNYLLTVSVPGWKEEELEIETVGGNLNITGKHTEETVEDQTHWIYRGIRK    100

STM1250          35 -----PYFLPK----GRPKAALGLIM----------------------EKG     54
                         .:.||:    ...|...||::                     .|.
AgsA            101 ADFQLSFSLPEHAKVNNAKLEQGLLLVEIYQEIPESEKPKKIAIESKPKA    150

STM1250          55 VGHCHNQPVYINAIDNHYQFGWWRKIVKMKGQRKWQ     90
                    :.|..
AgsA            151 IEHKS-------------------------------    155
```

Figure D98. Pairwise alignment of STM1250 and AgsA. Amino acid sequences were obtained from the NCBI database using the *S.* Typhimurium LT2 genome. Alignment was performed using EMBOSS needle.

Appendix E. STM1250 structure prediction (PHYRE)

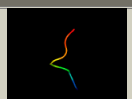
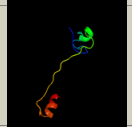
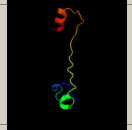
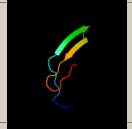
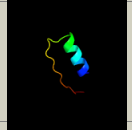
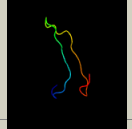
#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c1g0yl_	Alignment		31.6	67	**PDB header:**immune system **Chain:** I: **PDB Molecule:**antagonist peptide af10847; **PDBTitle:** il-1 receptor type 1 complexed with antagonist peptide af10847
2	d2i15a1	Alignment		24.8	32	**Fold:**MG296-like **Superfamily:**MG296-like **Family:**MG296-like
3	c5oeiA_	Alignment		21.9	14	**PDB header:**transport protein **Chain:** A: **PDB Molecule:**uncharacterized protein family upf0065:tat pathway signal; **PDBTitle:** r. palustris rpa4515 with oxoadipate
4	c2dvzA_	Alignment		14.8	14	**PDB header:**transport protein **Chain:** A: **PDB Molecule:**putative exported protein; **PDBTitle:** structure of a periplasmic transporter
5	c2f5xC_	Alignment		14.1	18	**PDB header:**transport protein **Chain:** C: **PDB Molecule:**bugd; **PDBTitle:** structure of periplasmic binding protein bugd
6	c6hkeB_	Alignment		12.8	16	**PDB header:**transport protein **Chain:** B: **PDB Molecule:**possible tctc subunit of the tripartite tricarboxylate **PDBTitle:** matc (rpa3494) from rhodopseudomonas palustris with bound malate
7	c4q51A_	Alignment		11.5	30	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**uncharacterized protein; **PDBTitle:** crystal structure of a putative molybdenum cofactor biosynthesis2 protein f from burkholderia cenocepacia j2315
8	c2lcyA_	Alignment		10.5	41	**PDB header:**viral protein **Chain:** A: **PDB Molecule:**virion spike glycoprotein; **PDBTitle:** nmr structure of the complete internal fusion loop from ebolavirus gp22 at ph 5.5
9	c1eqpA_	Alignment		10.4	38	**PDB header:**proteinase inhibitor **Chain:** A: **PDB Molecule:**eglin-c; **PDBTitle:** proteinase inhibitor eglin c with hydrolysed reactive center
10	c5e9uB_	Alignment		9.7	7	**PDB header:**transferase/chaperone **Chain:** B: **PDB Molecule:**glycosyltransferase-stabilizing protein gtf2; **PDBTitle:** crystal structure of gtfa/b complex bound to udp and glcnac
11	c2qpqC_	Alignment		9.4	16	**PDB header:**transport protein **Chain:** C: **PDB Molecule:**protein bug27; **PDBTitle:** structure of bug27 from bordetella pertussis

Figure E1. Phyre2 output for structural modelling of STM1250.