Extracytoplasmic stress response systems in *S.* Typhimurium

Claire Lewis BSc MRes

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Institute of Comparative Medicine
Faculty of Veterinary Medicine
University of Glasgow

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Statement

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Signed:

Claire Lewis

Abstract

Salmonella species can cause wide-ranging disease from mild food-poisoning enteritis to a systemic, sometimes fatal typhoid infection.

These bacteria have evolved to survive in different environments within and outside the host and do so through the regulation of differential gene expression following activation of certain stress response systems.

In gram negative bacteria such as *Salmonella*, envelope stress responses (ESR) are response systems that target stresses affecting components of the cell envelope such as the periplasm and outer membrane proteins. The two best characterised ESRs are the RpoE stress response system and the CpxAR two-component signal transduction system. Two further ESRs, the BaeSR response and the phage shock response have also recently been identified.

The intention of this thesis was to characterise the ESR systems of *S.* Typhimurium to widen our current knowledge of genes involved in these systems and their role in the pathogenesis of *S.* Typhimurium with the ultimate aim of identifying possible candidate vaccine genes that may be used in future therapeutics against *Salmonella* infection.

Firstly, extensive mutagenesis and phenotypic analysis studies were undertaken to characterise genes thought to be members of the RpoE regulon.

Study of the phage shock response was initiated through mutagenesis, characterisation and regulation studies.

A microarray experiment was designed in collaboration with colleagues at the Sanger Centre to identify members of the *S.* Typhimurium CpxAR regulon, with several members of this regulon being characterised further.

The structural components of HtrA, an important ESR protein in *S.* Typhimurium, were analysed and finally work within this thesis was involved in the investigation of potential overlaps between both the RpoE and CpxAR systems.

This led to the establishment of preliminary studies to investigate the vaccine potential of the *tol - pal* genes in *S.* Typhimurium.

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Finally I would like to thank my daughter Georgia and my imminently due second child for being constant reminders of the priorities in life.

I dedicate this to my sister Sarah who is always in my thoughts.

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Chapter One Introduction

1.1 Salmonella introduction

Salmonella species are gram-negative motile rods that can infect both humans and animals causing wide-ranging disease from mild food-poisoning (enteritis) to a systemic, sometimes fatal typhoid infection (254, 336).

There are at present 2,463 serotypes (serovars) of *Salmonella*, incorporating complex systems of nomenclature. This review will adopt the recently updated nomenclature system used at CDC that is based on recommendations from the WHO Collaborating centre. The genus *Salmonella* comprises two species, each of which has multiple serotypes: the two species are *S. enterica* and *S. bongori*.

1.2 *Salmonella* host specificity and disease

Salmonella species can be either host specific, or infect multiple host types causing different disease pathologies.

S. Typhimurium (the most common serovar) has a broad host range and its clinical symptoms depend on the host species involved; it causes salmonellosis (*Salmonella* gastroenteritis) in the human host and typhoid like disease in the murine host. Approximately 95% of human *Salmonella* infections are food borne with *S.* Typhimurium bacteria being acquired from contaminated food such as poultry, eggs and water (242). Once the bacteria invade the body, they can multiply and invade the intestinal mucosa leading to abdominal pain, cramps, diarrhoea and fever. Although most adult patients recover, fluid loss can be problematic in young children, the elderly and those that are immuno-compromised. An additional concern is the recent emergence of multi drug resistant strains of *Salmonella* (96, 279, 285, 336). A recent

review article discusses nontyphoidal multi drug resistance, current treatment protocols and the genetics of *Salmonella* drug resistance (6).

S. Typhi is host specific, infecting humans and higher primates, and it was a leading cause of death in the developed world until it was brought under control by extensive public health measures however, due to poor sanitation, inadequate healthcare poverty and overcrowding it is still a major cause of mortality in the developing world. Globally, there are an estimated 20 million cases per year, and 200,000 deaths associated with Typhoidal *Salmonella* serovars (61). Although antibiotic treatment can reduce mortality to less than 1%, the emergence of multi drug resistant strains is becoming a serious issue. *S.* Typhi bacteria are acquired by ingestion of food or water contaminated by the faeces of infected hosts. Following invasion, the bacteria can colonise the small intestine, penetrate the epithelium and spread to the lymphoid tissue, blood and gall bladder. Severe disease manifestations can occur such as haemorrhagic necrosis of the Peyer's Patches (PP) of the distal ileum resulting in tissue perforation, peritonitis, septicaemia and death (73, 96, 279, 285, 286).

Although our understanding of the molecular mechanisms of severe typhoid fever is limited, much has been learnt from the study of animal model systems (171, 336). As mentioned, *S.* Typhimurium can infect a murine host to cause a severe, systemic disease, similar to that of *S.* Typhi in the human host and as such has become the best-characterised animal model of typhoid fever. This model system has been used to study the many host-pathogen interactions that occur during the development of systemic disease and has shown that the pathogenesis of *Salmonella* infection relies upon efficient methods of bacterial invasion, survival and replication. The host-cell cytoskeleton is intricately involved in initial bacterial invasion, following which, *Salmonella* survive and replicate within vacuoles (336, 337).

S. Pullorum and *S*. Gallinarum are both host specific to poultry, though they result in different disease manifestations with *S*. Pullorum being a fowl-adapted strain that causes dysentery (Pullorum disease) and *S*. Gallinarum resulting in fowl typhoid (345).

S. Enteritidis is a non-host specific *Salmonella* species that can infect humans resulting in gastroenteritis. It is the most common cause of *Salmonella*-related food poisoning in the UK, and it has become a global problem infecting both poultry and the human host. The route to human host infection begins with colonisation, survival and multiplication of this pathogen resulting in contamination of eggs (134).

The availability of complete microbial genome sequence data has allowed the development of genomic-based approaches to investigate mechanisms of bacterial pathogenicity. More specifically, comparative genomics can be used to discover new virulence determinants and new vaccine targets (361, 395). Genome sequence data can be used alongside tools such as DNA microarrays to investigate the expression profiles of bacterial genes under different environmental situations (279, 361).

The genome sequence of a number of *Salmonella* strains have already been published and many more are in progress, this will further our understanding of the differences between host specific and non-specific *Salmonella*.

1.3 *Salmonella* pathogenesis

An understanding of how *Salmonella* can infect the human host and the host response to this infection is crucial to the management of these bacteria and the development of efficacious vaccines to bring these infections under control. As mentioned earlier, the pathogenesis of *Salmonella* infection can be divided into two main groups; one

mainly resulting in systemic infection and one resulting in a gastroenteritis type infection, only producing systemic infection in particular circumstances, such as in the very young or following viral infection.

To induce enteritis, *Salmonella* invade epithelial cells by bacterial mediated endocytosis (111) involving cytoskeletal rearrangement resulting in membrane ruffling from the cell surface which internalises the adherent bacteria.

In mice, which can be infected with *S*. Typhimurium causing a systemic infection, *Salmonella* preferentially adhere to microfold cells (M cells) of the intestinal epithelium as well as invading non phagocytic enterocytes (118). Enteritis associated *Salmonella* serotypes induce a secretory response in the intestinal epithelium and initiate movement of neutrophils into the intestinal lumen (118). Following passage across the intestinal epithelium *Salmonella* encounter the submucosal macrophage.

For the development of systemic infection to occur, *Salmonella* serotypes enter macrophages by macropinocytosis whereby they can evade microbicidal functions of the phagocyte by activation of virulence determinants that promote survival and replication in the intracellular environment (7, 279).

In order to colonise the host, *Salmonella* have various virulence determinants that must be in place.

An important aspect of *Salmonella* pathogenicity is its ability to survive within the host in the presence of innate immune effectors and extreme environmental conditions such as the low pH of the gut. Little is known about how *Salmonella* can adapt to survive in different environments, though a variety of virulence genes have been identified (128, 279, 374).

Many virulence genes are contained within *Salmonella* pathogenicity islands (SPI), which are centred in specific areas of the chromosome. A recent study has been

involved in using interpolated variable order motifs (IVOM) for the characterisation of horizontally acquired DNA. This method was applied to *S.* Typhi CT18 and predicted the presence of three novel SPIs, SPI-15, SPI-16 and SPI-17, as well as the previously characterised SPIs 1 to 10 (376). SPI-11 and SPI-12 are present in S. Choleraesuis and SPI-13 and SPI-14 are found in S.Gallinarum. SPI-1 and SPI-2 are known to encode type III secretion systems (TTSS).

TTSS are virulence determinants that are involved in the translocation of bacterial virulence proteins directly into the host-cell cytoplasm (163). These proteins can interfere with important host-cell functions including signal transduction, cytoskeletal arrangement, membrane trafficking and gene expression. In order for these proteins to be translocated into the host-cell cytoplasm they must cross the bacterial inner and outer membrane and the plasma membrane. This process occurs by the assembly of TTSS components to form a channel that by-passes the inner and outer membrane, called the needle complex(279). This TTSS is seen in many gram negative pathogens though the effector proteins translocated across the host cell are very specific to the pathogen involved (279). The effector proteins are encoded by genes that lie either in the same pathogenicity island as that encoding the secretion apparatus, or they can be elsewhere in the chromosome, such as on bacteriophages which is the case for a number of *Salmonella* TTSS targets such as *SopE* (251).

It is thought that *Salmonella* acquired these pathogenicity islands to allow the organism to diverge and adapt to new niches within the vertebrate host and also changing environmental pressures (105).

1.3.1 *Salmonella* pathogenicity island -1

SPI-1 is a 40KB DNA segment located at centisome 63 of the S. Typhimurium chromosome that encodes a TTSS responsible for the translocation of effector proteins into the cytosol of host cells necessary for invasion of non-phagocytic host cells (as shown in Figure 1.1). It is thought its primary role is the invasion of intestinal epithelial cells (105, 336). SPI-1 is known to be involved in the oral route of infection as mutants in the SPI-1 secretion apparatus show reduced virulence in oral models of infection however these same mutants do not affect the virulence of systemic models of infection(57). It is also known that all disease associated *Salmonella* serovars have an entire SPI-1 region, whilst some environmental serovars have deletions in their SPI-1 region that could be regarded as further evidence for the virulence status of SPI-1 (126). As outlined earlier, the proteins encoded by SPI-1 assemble to form a needle like complex that traverses the inner and outer bacterial membranes (199). In addition to these proteins, there are other proteins, known as effector proteins, encoded within SPI-1 that can be secreted and have the capability of altering eukaryotic cell structure (8). The needle complex delivers these secreted proteins to the epithelial cell cytoplasm when the bacteria are in close contact with the intestinal tract epithelial cells. Following this, the secreted proteins can alter the cytoskeletal structure and induce actin filament rearrangement of the associated bacteria (115).

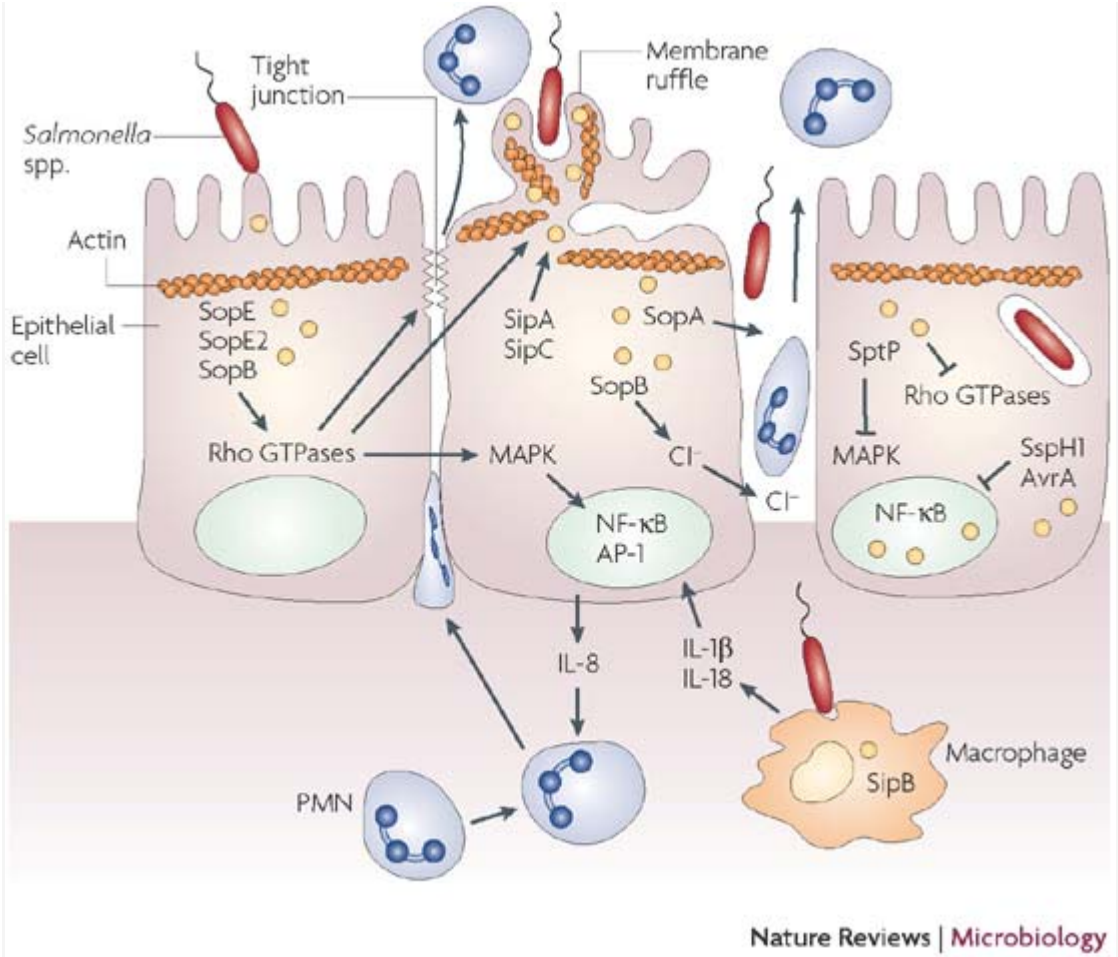


Figure 1.1 SPI-1 TTSS. On contact with the epithelial cell, salmonellae assemble the *Salmonella* pathogenicity island 1(SPI1)-encoded type III secretion system (T3SS) and translocate effectors (yellow spheres) into the eukaryotic cytoplasm. Effectors, such as SopE, SopE2 and SopB, then activate host Rho GTPases, which results in the rearrangement of the actin cytoskeleton into membrane ruffles, induction of mitogen-activated protein kinase (MAPK) pathways and destabilization of tight junctions. Changes in the actin cytoskeleton, which are further modulated by the actin-binding proteins SipA and SipC, lead to bacterial uptake. (144)

The Sip A, B and C proteins are a group of proteins whose translocation to the host cytosol is carried out via the SPI-1 TTSS. SipC is involved in actin nucleation and bundling of actin filaments *in vitro*, resulting in *in vivo* cytoskeletal rearrangement (239). SipB, SipC and SipD are known to be involved in invasion (55) and are involved in the translocation of other effector proteins such as SopB, SopE, SptP and AvrA (113, 118, 145, 146, 398).

SipA binds to F-actin, inhibiting depolymerisation to enhance the efficacy of SipC and SipB binds and activates Caspase I to allow it to cleave and activate IL-1β(406). Caspase I is involved in the apoptosis of *Salmonella* infected macrophages (156, 239). Although there are many virulence genes associated with *Salmonella*, it is important to have regulatory systems in place to co-ordinate the expression of these genes at the appropriate time.

SPI-1 is primarily concerned with control of invasion with SPI-1 encoded proteins and effectors being controlled by numerous regulators. All of these associated regulators are transcriptional regulators that activate invasion and can control genes that are either inside or outside the island (8).

The central regulator of this system is HilA which is a member of the ToxR/OmpR family which activates the *sip* operon and also the *inv/spa* and *prg* operons which encode components of the secretion apparatus (8, 14, 70, 89, 225). Other regulators of SPI-1 are SprB, HilC and HilD and InvF (225, 226). In addition to control of SPI-1 invasion by regulators within SPI-1, it is also under the positive and negative control of regulators outwith SPI-1. These global regulators can also be involved in the control of other non-invasion associated genes indicating that control of these invasion genes has been incorporated into existing regulatory pathways following the bacterial acquisition of SPI-1 by horizontal gene transfer (8). BarA/SirA is one such

example: it is a two-component regulator that is known to control invasion and loss of either BarA or SirA has been shown to greatly reduce invasion. BarA can regulate the expression of *hilA* and can also regulate other SPI-1 genes in a *hilA* independent manner (10, 183). It has been discovered recently that at least some control of invasion by BarA/SirA is controlled by a second regulatory system called the Csr system, which comprises a small protein called CsrA that acts post-transcriptionally. This protein was first identified in *E. coli* and binds to target messages in the ribosome binding site, altering their expression (328). CsrA is required for SPI-1 gene expression in *Salmonella*, and can also reduce the expression of invasion genes when it is over-expressed which illustrates a tight mechanism for control of invasion gene expression (9).

Other recently identified regulators of SPI-1 invasion genes include *rtsA* (92) which induces DsbA, a periplasmic disulfide bond isomerase required for SPI-1 TTSS and FliZ, a regulator of flagella production (227).

In addition to regulation by components within the SPI-1, and global regulators external to SPI-1, invasion is also known to be controlled by small signalling molecules such as ppGpp. ppGpp is produced in response to *relA* and *spoT* activity, with expression of ppGpp resulting in a reduction in RNA synthesis. Mutants deficient in *relA* and *spoT*, and hence deficient in ppGpp fail to express invasion genes and are avirulent in mice (296). This activity is not unique to *Salmonella*, as ppGpp is also known to induce virulence in *Legionella pneumophila* (262).

It is important for *Salmonella* to be able repress expression of invasion genes, as expression of invasion genes within epithelial cells for example, would be inefficient to the bacterium as invasion has already occurred at this point. One interesting negative regulator of SPI-1 gene expression is the PhoP/PhoQ two-component

regulator. The PhoP/PhoQ regulator is essential to the expression of SPI-2 genes, which will be discussed later in this introduction. SPI-2 is required for bacterial growth in epithelial cells following invasion and it is also required for survival in macrophages (53, 336). As such, it makes sense that this regulator can also repress genes no longer needed for invasion. The negative repression of SPI-1 genes by PhoP/PhoQ is mediated by *hilA* and is an interesting example of a two-component regulator that can repress SPI-1 genes when they are no longer required, while also activating expression of genes involved in the SPI-2 system (18, 98). Other negative regulators of SPI-1 include Hha, a histone-like protein that instigates repression through its binding to HilA (99), and H-NS, a small nucleoid-binding protein (338).

In addition to the genetic components involved in repression of SPI-1 invasion genes, environmental factors can also play a role. An example of one such environmental factor is bile production which represses genes of the SPI-1 system. Bile is secreted into the proximal small intestine where invasion does not occur and as such is involved in the repression of genes not required at that location (305).

1.3.2 *Salmonella* pathogenicity island – 2

SPI-2 is a 40 KB segment of DNA located between centisome 30 and 31and is known to encode a second TTSS (shown in Figure 1.2). This SPI is required for virulence and proliferation within macrophages and other cells (154). SPI-2 comprises 31 genes organised into four operons. Two operons encode secretion apparatus genes (*ssa*), one operon encodes secretion system effectors and chaperones (*sse* and *ssc*) and the final operon encodes SsrAB, a two-component regulatory system (344). SPI-2 is required for bacterial growth in epithelial cells and survival in macrophages, and activates the

replication of intracellular bacteria within membrane bound *Salmonella*-containing vacuoles (SCV's) (53, 279, 336, 374). The SPI-2 TTSS is activated following invasion of eukaryotic cells and it has been shown to activate bacterial growth in many different cell types (27, 53, 154, 277). SPI-2 is involved in the inhibition of many diverse activities such as fusion between SCV's and lysosomes (371), avoidance of macrophage killing (117), maintenance of the SCV membrane (26) and cholesterol accumulation around the SCV (46). There are several effector proteins that have been identified both within and outside SPI-2 that are involved in the inhibition of these activities. SpiC is located within SPI-2 and was the first protein to be identified as a SPI-2 effector protein. This protein is exported by the *ssa* TTSS into the host cell cytosol where it interferes with intracellular trafficking and a null mutant of SpiC resulted in decreased virulence, indicating that inhibition of intracellular trafficking by *Salmonella* is essential to its pathogenicity (371). SseF and SseG are two other SPI-2 effector proteins contained within SPI-2. Mutations in these genes resulted in slight macrophage replication defects, and also a slight reduction in virulence in mice (154). Interestingly, *sseF* and *sseG* mutants in *S.* Typhimurium SL1344 background strain were severely defective in Sif (*Salmonella*-induced filament) production in epithelial cells (137), yet these mutations in the *S.* Typhimurium 12023 background strain allowed the formation of morphologically abnormal Sifs (200). This observation highlights the disparities that can occur between strains. In addition to effectors contained within SPI-2, there are many secreted effectors that are encoded outside SPI-2. SseJ is one such protein that is involved in the membrane dynamics of SCV's. It is encoded by a gene located on a phage and *sseJ* mutations result in slight virulence attenuation during systemic

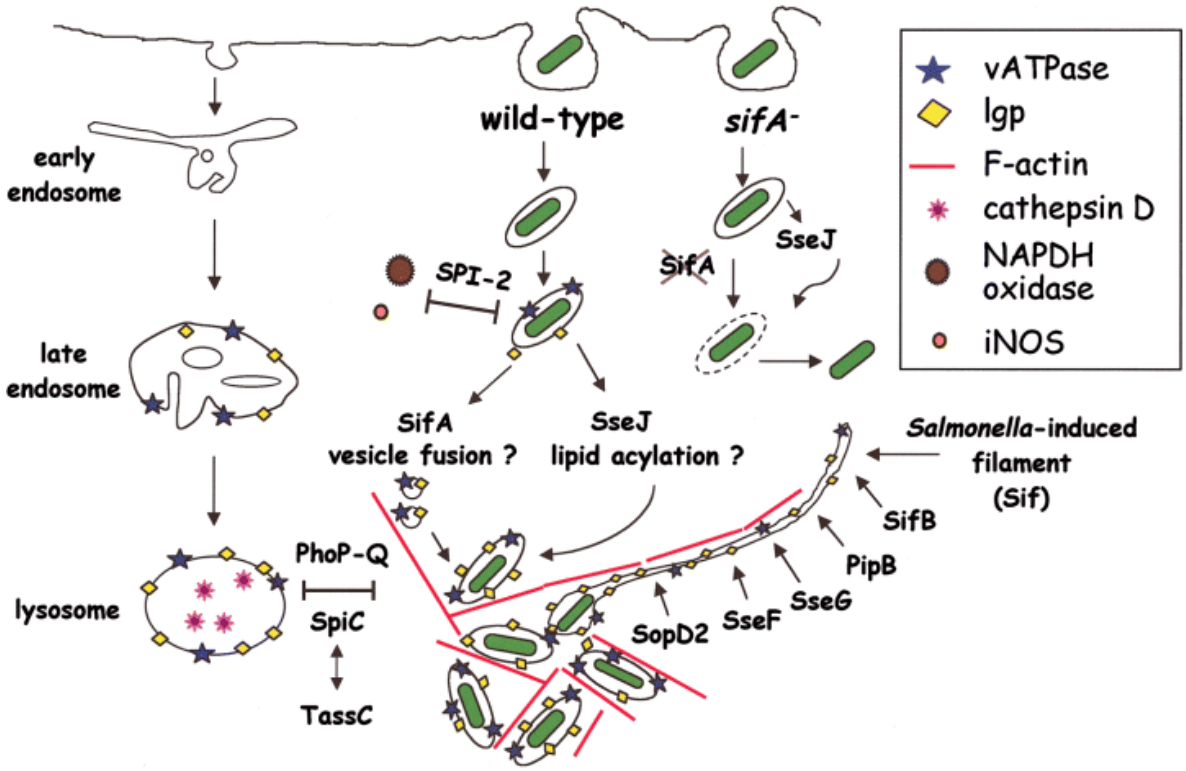


Figure 1.2. SPI-2 TTSS. The phenotypes of wild-type and *sifA* mutant *S. typhimurium* (green) are shown in relation to different host cell proteins (indicated in box) and vesicular compartments. SCVs do not interact extensively with late endosomes or lysosomes. In macrophages, conflicting evidence implicates the SPI-2 effector protein SpiC (which interacts with the host protein TassC) and the PhoP-Q regulon in this process. SifA, SifB, SseJ, SseF, SseG, PipB and SopD2 are examples of SPI-2 translocated proteins that localize to the SCV and Sifs in epithelial cells. Loss of vacuolar membrane from the *sifA* mutant requires the action of SseJ.(389)

infection of mice, and also slightly reduced levels of replication in primary macrophages (112, 334).

As with the SPI-1 system, SPI-2 gene expression has also been shown to be affected by global regulators within *Salmonella*, including PhoPQ and OmpR/EnvZ (79, 207). As mentioned earlier, PhoPQ is a two-component regulatory system that is essential to SPI-2 gene expression which also plays a role in the inhibition of SPI-1 expression. A recent study has shown there to be an overlap in virulence control between the Rcs signal transduction system in *S.* Typhimurium and the PhoPQ regulatory system. Rcs was first characterised in *E. coli* as a regulator of colonic acid and capsule synthesis. (121). The PhoPQ system is present in non-pathogenic bacteria such as *E. coli* K12, however it plays an important role in the virulence of *Salmonella* (263). The PhoPQ system of *Salmonella* controls expression of over 40 proteins including MgtA and MgtBC which together comprise two of the three Mg2+ uptake systems in *S.* Typhimurium (351). PhoPQ is also involved in the regulation of *pagC*, a virulence gene known to be involved in macrophage survival and mutants of PhoP and / or PhoQ have been shown to be attenuated for virulence (120, 247, 248). These observations all support the theory that PhoPQ is a regulatory system in *Salmonella* essential for virulence which has been shown to be important in survival and replication within macrophages (119, 246, 247). It should be noted that SPI-1 and SPI-2 are not exclusively involved in pre- and post-invasion respectively.

As shown, SPI-1 and SPI-2 encode TTSS's and are crucial to the virulence of *Salmonella*, however the virulence phenotype of the remaining SPI's is still under investigation.

SPI-3 is a 13KB segment of DNA with *mgtC* being the only SPI-3 gene to have a virulence phenotype. *mgtC* is thought to be required for survival in low Mg2+ and

low pH conditions such as those in the intracellular vacuolar environment and as such is essential for invasion of host cells (41, 105).

1.4 *Salmonella* and the immune response

S. Typhi infections pose a serious health risk to developing countries whilst other *Salmonella* serovars including Typhimurium and Enteritidis remain a common cause of food-borne gastroenteritis. In order to establish infection the *Salmonella* bacterium must first overcome the varying mechanisms of immunity of the host. The severity of infection is dependent on both the virulence of the infective organism as well as the immune response elicited by the host (233).

Salmonella serovars associated with the development of gastroenteritis involve mass intestinal colonisation while host-specific serotypes causing systemic disease such as *S.* Typhi, are involved with colonisation of monocyte / macrophage cells (16, 372). Much of our current understanding of the response of *Salmonella* to the host immune system has come from studies of mice infected with *S.* Typhimurium. Infection of the murine host with *S.* Typhimurium results in systemic disease and as such I will discuss current thoughts on the host immune responses to systemic *Salmonella* infection.

Pathogen recognition by the host involves the toll like receptor (TLR) family of receptors which are transmembrane proteins used by the innate immune system to signal the presence of bacteria to the host and also the subsequent initiation of the host defence system (73). Activation of TLRs leads to the release of cytokines involved in the innate and adaptive immune response (124). There are now thought to be ten human toll like receptors, with different toll receptors recognising various ligands such as bacterial DNA, LPS, flagella or lipoproteins (319). The importance of TLR in

the host immune response is emphasised in recent studies that showed a vaccine containing bacterial ligands for TLR1, TLR2, TLR4, TLR6 and TLR9 administered to mice to give rapid, effective short term protection against *S*. Typhimurium (342).

TLR4 recognises LPS from gram-negative bacteria and has been shown to increase the production of inflammatory cytokines such as interleukin (IL)-1 and IL-6 (24, 267). It is one of the most studied mammalian TLRs and is expressed by macrophages, dendritic cells and B cells (42). LPS recognition by TLR4 is dependent upon TLR4 forming a complex with the secreted protein MD-2 (1) and upon stimulation by LPS, the TLR4-MD-2 complex up-regulates expression of various factors such as nuclear factor (NF)-κB and activating protein (AP)-1 target genes, including cytokines, such as IL-6, IL-8, tumor necrosis factor (TNF), and type I interferon, IFN-β (88).

Mice deficient in either TLR4 or MD-2 are susceptible to *S*. Typhimurium infection (375) while hosts carrying a TLR4 Asp299Gly allele are more susceptible to septic shock caused by gram negative bacteria (224).

Following TLR ligand recognition, a cascade of inflammatory responses is initiated with cytokines IFN-γ and TNF-α inducing the activation of macrophages and subsequent killing of the bacteria by induction of the macrophages bactericidal mechanisms. Macrophages phagocytize *S*. Typhimurium by receptor-mediated uptake following opsonisation of *Salmonella* with antibodies or complement (258, 265). IFN-γ (produced by natural killer cells) and TNF-α (produced by macrophages) are two cytokines essential to the primary stages of *Salmonella* infection (258, 396).

In addition to the TLR family, other innate immune genes include the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR). The NLRs are a family of proteins made of NOD and neuronal apoptosis inhibitor like (NALP) proteins

which play an important role in sensing microbial products and invading pathogens (4, 31). NODs activate transcription factors such as NFκB upon exposure to microbial products (290) while the NALP proteins are involved in the activation of IL-1β through their interaction with molecules such as caspase 1 and caspase 5 (232). Ipaf is an NALP like protein that is involved in *S*. Typhimurium induced caspase 1 activation (230).

Slclla 1 (previously known as Natural Resistance Associated Macrophage Protein -1 [NRAMP-1]) is one of the best studied host response factors. It is a divalent metal transporter (178) associated with innate resistance to intracellular pathogen infections and susceptibility to autoimmune disorders (192). Slclla is expressed in macrophages and has a crucial role in the host response to bacterial invasion. In particular it plays an important role in the ability of macrophages to control *S*. Typhimurium (378). Mouse strains that lack a functional Slclla 1 protein have a much higher susceptibility to infection due to their macrophages having a reduced ability to kill the *S*. Typhimurium cells (129, 378). In-bred mice that carry a mutant allele of *slclla* are often used in *S*. Typhimurium mouse model infection studies as these mice are unable to control a low infective dose of *Salmonella*, resulting in wide spread systemic infection (23, 379).

In summary of the innate system, the initial stages of *S*. Typhimurium infection involve the activation of phagocytes via inflammation of infected tissues and subsequent production of IFNγ by natural killer cells. At this stage, many of the bacteria are eliminated by the host however total loss of *S*. Typhimurium is not achieved through this innate immune response. The bacteria can adapt to the innate immune response by expression of various virulence factors that provide it with increased resistance to bactericidal activity (258) through regulation of gene

expression by regulatory systems such as the PhoPQ two-component regulatory system discussed earlier. Bacteria can also overcome the host defence by utilising the TTSS to inject bacterial proteins into the host cell, whereby the host cells signalling machinery can be altered (73).

Following the early stage of infection, bacterial growth is reduced by the onset of the adaptive host response. The adaptive host response relies on numerous cytokines (IFNγ, TNFα, IL-12, IL-15 and IL-18) as well as the involvement of inflammatory phagocytes in the infected tissue. The control of bacterial growth is achieved by production of reactive nitrogen intermediates (RNI) via the inducible nitric oxide synthase (iNOS) and also reactive oxygen intermediates (ROI). Subsequent clearance of the bacteria at a later stage in the infection requires the CD-28 dependent activation of CD4+ TCR αβ T cells and is controlled by MHC class II genes (233). The involvement of T cells in immunity against *Salmonella* has been extensively reviewed (303, 318, 357) .Many experiments have shown the CD4+ T cells to play a greater role than the CD-8 T cells with the depletion of CD4 T cells having a greater effect on the control of primary *Salmonella* infection (235) and it has also been shown that mice deficient in CD4+ T cells (MHC class II deficient mice) failed to clear infection with an attenuated strain of *S.* Typhimurium and developed chronic disease (157, 235). Various studies have shown that infection of mice with *Salmonella* induces a Th1 response which is characterised by production of large amounts of IFNγ (258, 294, 368) while other studies have shown that under specific conditions Th2 responses can be induced by infection with attenuated *Salmonella* resulting in production of IL-4 and increased serum levels of IgE (353). Following clearance of a *Salmonella* infection, an efficient Th1- type immunological memory and antibodies against *Salmonella* are necessary to prevent re-infection (233). Anti-salmonella IgM is

detected in serum early after infection, followed by a later IgG response (218, 244) while IgA has been detected in both humans and animals exposed to live *Salmonella* infection (110, 147, 266). *Salmonella* specific antibodies recognise numerous antigens such as O-polysaccharide and core regions of LPS, Vi, lipoproteins, heat shock proteins and flagella (40, 58, 147, 201, 241, 365).

To prevent re-infection of *Salmonella* infection, cytokines, T-cells and antibodies are all required (234) with T-cells and antibodies alone providing protection against only a modestly virulent strain of bacteria (90, 400, 401). The human typhoid vaccine based on S. Typhi Vi polysaccharide antigen (2, 194) induces an antibody response however it is not capable of inducing Th1 immunity which is thought to be one of the reasons for its moderate efficacy (147, 368).

These entire studies together highlight the intense pressure put on the invading *Salmonella* bacteria by the host immune response. To invade and proliferate, the bacteria must find ways to overcome these pressures.

1.5 Bacterial Stress Response

1.5.1 Bacterial Sigma factors and their role in stress response

To survive in a changing environment the bacterium must be able to regulate the expression of specific genes, which it does through the binding of RNA polymerase to a promoter to initiate transcription (32).

RNA polymerase is comprised of $\alpha 2\beta\beta'$ subunits combining to give a molecular mass of approximately 400 kilo Daltons. To initiate transcription, an additional sigma (σ)

subunit is required to bind to the core RNA polymerase forming a holoenzyme that can then interact with specific promoter elements that are situated 10 and 35 base pairs upstream (positions -10 and -35) of transcription-initiation points (32, 149).

Numerous sigma factors exist and these can be categorised as belonging to either the σ^{70} family or the σ^{54} family, which are two structurally unrelated sigma factor families. Table one shows known sigma factors in S. Typhimurium, including factors which are known to induce these factors.

1.5.1.1 σ^{70} responses

Most bacterial sigma factors belong to a homologous family closely related to σ^{70}, with distinct regions of closely conserved sequence. Primary sigma factors direct most transcription during log phase growth however bacteria can also produce alternative sigma factors that can be transcribed when environmental conditions require a change of gene expression within the cell. The reversible binding of alternative sigma factors allows formation of different holoenzymes able to distinguish groups of promoters required for different cellular functions (151, 283).

The regulon controlled by a particular sigma factor can contain hundreds of genes.

The group of genes controlled by any one bacterial σ factor can have a very specific function as is seen in the case of the sporulation sigma factors in *Bacillus subtilis* (295) or it can control the regulation of genes involved in multiple functions. σ^{70}, the most common bacterial promoter, for example governs the regulation of many genes with diverse functions.

Alternative sigma factors are now known to have specific roles in regulating expression of virulence genes in bacterial pathogens and many are involved in the

stress response of the bacteria. The σ^E, σ^H, and σ^N alternative stress response sigma factors will be discussed in more detail later in this review.

B. subtilis σ^B was one of the earliest bacterial alternative sigma factors identified (140, 141). This sigma factor is present in other gram positive bacterial species including *L. monocytogenes* and *S. aureus*, and its activity is known to increase in response to acid, ethanol and heat exposure (21, 48, 104). σ^B of *L. monocytogenes* is an example of a stress response sigma factor that is involved in the regulation of genes during stationary phase, and also general stress response genes (189).

σ^S in gram negative bacteria such as *E. coli, Pseudomonas. aeruginosa*, and *S.* Typhimurium is responsible for both stationary-phase and stress response gene expression in a similar way as is seen for σ^B control of genes in gram positive bacteria. The expression of the σ^S regulon is very complex and involves transcriptional, translational and posttranslational mechanisms which are reviewed more fully in (152).

Table One. *S.* Typhimurium alternative sigma factors

Family	Sigma factor (also known as)	Inducers include
σ^{70}	σ^E (σ^{24})	High temperature, periplasmic protein folding disruption
	σ^H (σ^{32})	High temperature
	σ^S (σ^{38})	Stationary phase, nutrient deprivation
	fliA(σ^{28})	
σ^{54}	σ^N (σ^{54})	Nitrogen limitation

It is thought that σ^S is responsible in an in direct fashion for the pathogenesis of *E. coli* species. For example, studies investigating *E. coli*σ^S mutants have shown these mutants to have decreased acid resistance and faecal shedding in mice and cattle (304) which ties in with the knowledge that the *E. coli* strain 0161:H7 is acid resistant. Environmental stress conditions such as osmotic shock, heat and low pH are thought to induce σ^S and σ^S mutants have shown reduced survival under similar environmental conditions (13, 62, 123, 153, 390).

S. Typhimurium σ^S is similar in both function and regulation to *E. coli* σ^S however unlike *E. coli* there have been numerous studies showing the direct dependence of *S.* Typhimurium on σ^S for full virulence. *S.* Typhimurium σ^S controls a regulon of 30 or more genes and plays an important role in the survival of *S.* Typhimurium following sudden encounters with a variety of stress conditions or upon entry into stationary phase (151, 203). Reduced availability of essential nutrients such as carbon is an important environmental stress which *Salmonella* can encounter. The starvation stress response (SSR) is mounted following such nutrient deprivation and many of the genes regulated by the SSR have been identified. Most of these genes are positively or negatively controlled by induction of σ^S and also the cAMP: CRP complex (354). SSR regulated genes are involved in long term survival and a recent study has confirmed that σ^S and σ^E regulated genes are required for optimal *S.* Typhimurium growth during high osmolarity conditions and long-term survival during starvation at cold shock and at 37ºC (240).

In *S.* Typhimurium, σ^S controls expression of the *Salmonella* virulence plasmid genes (*spv*) that are required for systemic infection of the host (100, 173). Studies have

shown an σ^S mutant to be 10 times less virulent than an *rpoS*$^+$ spv-negative strain and σ^S mutants in *S.* Typhimurium have been shown to have a reduced ability to colonise both the spleens and Peyers patches of infected mice (136, 270). In addition to this, other studies have shown an σ^S *aroA* strain to have a greater degree of virulence attenuation than is seen in an *aroA* strain alone (60). *S.* Typhi has no virulence plasmid and as such the role of σ^S in these serovars in undefined (60, 191).

A study was performed to identify σ^S regulated genes in *Salmonella* with the use of lacZ transcriptional fusions using the transposon Tn5B21 transposon. This study identified 38 unique σ^S regulated genes. Of these genes, fourteen mapped to genes present in both *Salmonella* and *E. coli* and ten genes were identified as new members of the σ^S regulon in *S.* Typhimurium (60, 173).

P. aeruginosa is also controlled by σ^S. Under certain circumstances σ^S can either positively or negatively regulate toxin expression allowing it to contribute directly to its pathogenesis. In a *P. aeruginosa* σ^S mutant exotoxin A and alginate production is reduced by 50% compared to that of wild type yet other studies have shown reduced RpoS expression and σ^S loss to cause increased expression of pyocyanin, an antibiotic involved in lymphocyte proliferation (197, 362).

An important effect associated with loss of σ^S is the reduced expression of quorum sensing systems, which are important *P. aeruginosa* virulence factors. This is highlighted in studies that show σ^S to be involved in expression of genes regulated by the *P. aeruginosa rhl* (RNA helicase) and *las* (a transcriptional regulator) quorum-sensing systems (292, 394).

Virulence factors that are regulated by the *rhl* and *las* quorum-sensing systems include aminopeptidase, endoproteinase, lipase (275) and lectins (341). Quorum-sensing mutants have been shown to be less virulent in mouse and rat infection studies

which demonstrates an in direct effect upon virulence by σ^S (209, 291, 409). However when interpreting the results of animal infection studies it should be noted that the role of σ^S in *P. aeruginosa* is very dependent on the model system being tested. For example, although an σ^S mutant showed no virulence defects in a rat chronic lung model compared to wild type, another study found an σ^S mutant to be half as virulent as wild type in *Caenorhabditis elegans* (352, 362).

Other important bacterial sigma factors include the σ^{28} family. This family of sigma factors is present in both gram positive and gram negative bacteria and its primary role is involved in transcribing genes involved in both flagellar synthesis and bacterial motility (148, 249).

FliA of *S*. Typhimurium is a σ^{28} factor involved in flagellar synthesis and the flagella of *S*. Typhimurium have been shown in various studies to have a role in virulence (44, 223). The structure, assembly and function of *S*. Typhimurium flagella is a complex process involving many genes and operons, with the expression of flagella known to be controlled in a multi-faceted manner. flhDC is known as a class I operon that encodes activators for transcription of the class II operon. FliA is a member of the class II operon which is responsible for expression of the class III genes (175, 280) and the FlgM gene product is an anti-sigma factor that is responsible for the negative regulation of flagellar synthesis via inhibition of *fliA* (164).

fliA, encoding σ^{28}, is also present in *Yersinia enterocolitica*, where regulation of flagellar gene expression is similar to that in S. Typhimurium. In *Y. enterocolitica*, FlhC and FlhD are regulators of expression of genes involved in flagellar synthesis (177).

1.5.1.2 σ^{54} stress response

As previously mentioned, sigma factors can belong to either the σ^{70} family discussed earlier or the σ^{54} family, which is also called σ^{N} in most species. σ^{N} is present in many species and can control a variety of different processes including nitrogen metabolism. The different processes controlled by σ^{N} vary between species and are often involved in systems associated with virulence. For example, σ^{N} in *Helicobacter pylori, Campylobacter jejuni*, and *Vibrio cholerae* controls the flagella virulence mechanism through control of the class II flagellar genes (179, 271, 307), it controls the TTSS genes *hrpL* in *P. syringae* and *Erwinia carotovora* (50, 172), and it controls the alginate virulence genes *algD*, and *algC* in *P. aeruginosa* (35, 410).

The role of σ^{N} in the virulence of *P. aeruginosa* has been well studied, and it is understood to play a key role in the pathogenesis of this organism.

σ^{N} is involved in the regulation of alginate which is known to be a virulence factor important in *P. aeruginosa* strains that can colonise the lungs of cystic fibrosis (CF) patients (35, 410). It is also involved in the regulation of both the flagella (71) and pili systems (369) in *P. aeruginosa* which are also known to be associated with the virulence of this organism. *P. aeruginosa* null σ^{N} mutants produce neither pilin nor form pili which are involved in adhesion to host cells and internalisation (369). In addition to this loss of pili production, σ^{N} null mutants are also less capable of invading host cells compared to wild type (298) and exhibit loss of flagellar motility resulting in a reduction in the virulence of these σ^{N} deficient strains (150, 369). This reduction in virulence associated with the loss of flagella has been shown in several studies of *P. aeruginosa* (85, 103, 264).

Proteus mirabilis is another bacterial species that exhibits reduced pathogenesis when σ^N is inactivated shown by a 1000 fold reduction in virulence compared to wild type, however inactivation of in σ^N *P. mirabilis* shows no observable difference in growth or fimbrial production compared to wild type (402).

Regulation of the PspA operon (an important stress response system discussed later) is known to be dependent on σ^N in *Y. enterocolitica*, and the PspA operon has been shown to be critical to the virulence of this species (237).

PspA has also been shown to be dependent on σ^N in *E. coli* (87) and additional microarray analysis and other studies have shown at least 100 *E. coli* σ^N dependent genes to be affected by nitrogen limitation which highlights the important role that nitrogen plays in this system (321).

An important aspect of σ^N -dependent transcription is its absolute requirement for a transcriptional activator. These activators bind to sites analogous to eukaryotic enhancers, hydrolyse ATP and interact with σ^N-containing RNA polymerase. As σ^N -dependent transcription is dependent upon an activator, its expression can be completely turned off giving a wide range of control from high to low expression (320).

Although σ^N is present in S. Typhimurium it does not appear to have the dramatic effect of pathogenesis as is seen in some of the other bacterial species described above. It is however involved in O-antigen expression in *S.* Typhi along with σ^S through control of *rfaH* promoter activity (30).

1.5.2 Cytoplasmic Stress Response

Bacteria respond to stresses with the use of many regulatory pathways, with specific pathways targeted to stress perceived by various components of the cell structure. The cytoplasmic stress response involves regulatory pathways that respond to stress within the cytoplasm. The classical heat shock response is the best-studied cytoplasmic stress response. σ^H is known as the classical heat shock sigma factor and has been shown to provide major protective functions against thermal stress.

The σ^H regulon of *E. coli* encodes chaperones, proteases and other heat shock proteins and this heat shock response system is present in many other bacteria including *S.* Typhimurium where it can also respond to increases in temperature by induction of heat shock proteins (254, 316).

On exposure to heat shock there is an increase in the cellular level of σ^H through enhanced synthesis and stability followed by the accumulation of abnormal proteins that sequester the chaperones DnaK and DnaJ away from σ^H allowing σ^H to bind to RNA polymerase initiating the activation of transcription of heat shock genes (216, 217, 316).

E. coli σ^H mutants are only able to grow at temperatures below 20ºC (408) however it has now been shown that multiple stresses such as ethanol, starvation and oxidative stress can also activate the heat shock response in *E. coli* and *Salmonella* species (316).

Recent studies have analysed the σ^H regulon using genome-wide expression analysis and promoter validation to identify new σ^H regulated genes. These studies identified 29 new σ^H dependent promoters and 57 new members of the σ^H regulon (273).

The overlapping OxyR and SoxRS regulatory pathways characterise another cytoplasmic stress response that is present in both *E. coli* and S. Typhimurium. These pathways detect oxidative damage in the cytoplasm and respond by inducing the synthesis of genes that encode antioxidant enzymes (102). *S.* Typhimurium encounters and must be able to respond to various sources of ROI including for example the high concentrations of superoxide present within macrophages. These large amounts of superoxide are produced by the eukaryotic cell to act as an anti-microbial defence (180).

Studies in *E. coli* investigating gene expression following superoxide stress have shown that expression levels of 112 genes are up or down regulated in response to paraquat (300) while a similar study showed 140 genes to be induced upon exposure to hydrogen peroxide (404).

In both *E. coli* and *Salmonella*, the SoxR/S regulon is activated upon intracellular exposure to superoxide (132, 301) and this regulon comprises 10 genes including *micF* which controls OmpF expression and *fur*, the ferric uptake repressor (403). The OxyR system is activated in response to hydrogen peroxide and this system is only active as a transcription factor in the oxidised state. OxyR controls the expression of genes such as *katG*, a catalase that can inactivate hydrogen peroxide, and also other genes whose expression protects against oxidative damage. *fur* is also regulated by the OxyR system which highlights the importance of efficient maintenance of intracellular iron levels (180, 403).

1.5.3 Extracytoplasmic Stress Response

Employment of σ^H is one way in which bacteria can respond to stress within the cytoplasm. It has now been established that bacteria can respond in a similar manner to periplasmic stress through its extracytoplasmic stress response (ESR). A recent review has investigated the role of the ESR in pathogenesis of gram-negative bacterial pathogens (332). The ability of bacteria to respond to extracytoplasmic stress came from studies of the *htrA* gene in *E. coli*. Firstly it was identified that a *htrA* mutant could not grow at high temperatures and was subsequently termed *htrA* for High Temperature Requirement (220). Initial studies showed that *htrA* encodes a periplasmic protease essential for growth at elevated temperatures above 42°C (221, 316, 360). At the same time another group identified the same mutant as failing to digest misfolded proteins in the periplasm and was named *degP* (360). I will refer to this gene as *htrA* throughout this thesis. As with other heat shock proteins, HtrA synthesis was shown to increase in response to heat shock at the transcriptional level. *In vitro* transcription studies showed the HtrA promoter to be transcribed by σ^E in association with the RNA polymerase core enzyme. These experiments confirmed the existence of a second heat shock regulon controlled by σ^E in place of σ^H and were the first evidence that the *E coli* σ^E factor belonged to the Extracytoplasmic factor (ECF) family (93). This was further substantiated with the finding that misfolding of transported proteins in general results in an σ^E-dependent response. HtrA, is now known to be a widely conserved heat shock protein controlled by both the σ^E and the cpxAR regulatory systems (56, 63, 65) and plays an important role in the virulence of

S. Typhimurium*, Streptococcus pneumoniae* and *Y. enterocolitica* (167, 174, 214, 284), while in *Shigella flexneri*, *htrA* is required for intracellular spread in monolayers of Henle and caco-2 cells (308).

A *S.* Typhimurium *htrA* mutant showed increased sensitivity to oxidative stress *in vivo* which replicated similar results seen in an *E. coli htrA* mutant (49, 182, 359) and a *S.* Typhimurium *htrA* mutant has been shown to be an effective live vaccine (47). Work within our laboratory has shown that the *S.* Typhimurium σ^E mutant was attenuated in mice and similar studies investigating *htrA* showed the *htrA* mutant to also be attenuated, however not to as high a degree as is seen in the σ^E mutant(167, 406). This research confirmed our presumption that there were rpoE-regulated genes other than *htrA* that play an important role in the virulence of *S.* Typhimurium and became the starting point of our studies to characterise both the σ^E and CpxAR regulons of *S.* Typhimurium (167, 331, 349). In the following sections I will discuss current knowledge on the four ESR systems present in S. Typhimurium: rpoE, CpxAR, BaeSR and the Phage shock protein response.

1.5.3.1 rpoE

The *rpoE* gene encodes σ^E which is an alternative sigma factor that controls the extracytoplasmic response in *E. coli* (74, 93, 310) S. Typhimurium and other bacterial species. A model of the regulation of σ^E in *E. coli* is shown in Figure 1.3.

σ^E can be activated by several inducing factors including heat, ethanol, periplasmic protein folding disruption, biofilm formation and over expression of outer membrane proteins (28, 243, 252, 256). *rpoE* is the first gene in a four gene operon comprising *rpoE, rseA, rseB* and *rseC* (75, 253). RseA is an inner membrane-bound anti-sigma

factor that negatively regulates σ^E by binding to σ^E and sequestering it from the core RNA polymerase. σ^E is also negatively regulated to a lesser degree by RseB which is a periplasmic protein that binds to RseA, enhancing its activity. The stability of RseA is further strengthened through the activity of two proteases DegS and RseP (also known as YaeL). This protein is autoinhibited in the absence of envelope stress (188, 385). Activation of DegS by envelope protein misfolding relieves the inhibition of DegS allowing it to cleave RseA. The final step in the release of RseA from the RseA:RpoE complex is instigated via the SspB protein that directs this complex to the ClpXP protease initiating RseA degradation (109). The activity and expression of *E. coli* σ^E has recently been reviewed by Alba (5) and various studies have been involved in the characterisation of the *E. coli* σ^E regulon. Initially, 10 genes were suggested as part of the σ^E regulon in *E. coli* by a study using 2-dimensional gel electrophoresis (310). Four of these genes were identified as *rpoH, rpoE, htrA* and *fkpA*, encoding a periplasmic peptidyl prolyl isomerase (63, 309, 310).

In 2001, Dartigalongue et al (66) further expanded the number of members of the σ^E regulon in *E. coli* to 43. Following on from this, a two-plasmid system was designed for the identification of promoters recognised by RNA polymerase containing the extracytoplasmic stress response σ^E in *E. coli* (15, 29). This system used two *E. coli* plasmids, pAC-rpoE4 and pSB40. pAC-rpoE4 contains the *E. coli rpoE* gene under the control of an arabinose inducible pBAD promoter. Induction by arabinose allows σ^E to accumulate in the cytoplasm where it can interact with RNA polymerase core enzyme to form $E\sigma^E$. $E\sigma^E$ can then recognise σ^E- cognate promoters from a library of fragments present in the second plasmid pSB40. In pSB40, these fragments are cloned upstream of a promoter-less lacZα reporter gene (322, 323).

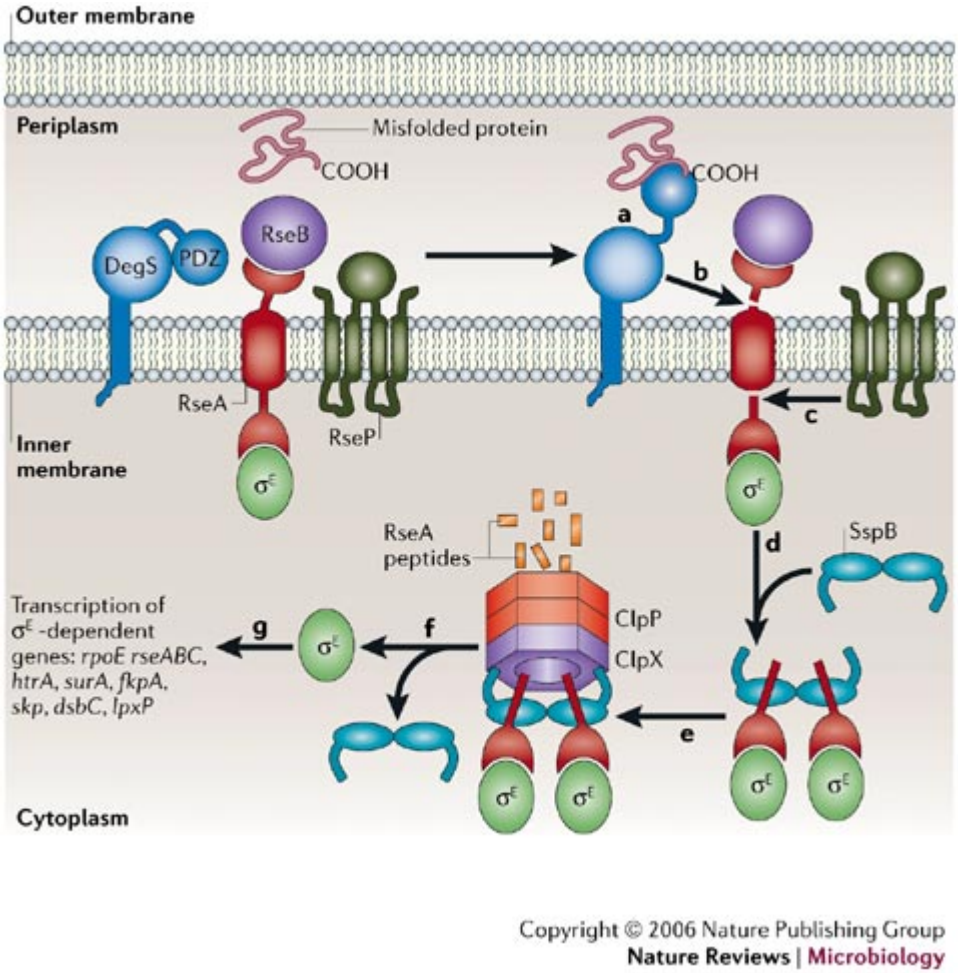


Figure 1.3 Model for regulation of σ^E activity in *E. coli*. (332)

This two-plasmid system identified 11 new rpoE-dependent promoters that could direct the expression of 15 new σ^E genes: *fusA, tufA, lpxP (ddg), psd, sixA, bacA, sbmA, yaiW, smpA, yeaY, ybaB, recR, yiiS, yiiT* and *yfeY* (66, 323). *E. coli rpoE* expression is controlled by two promoters P1 and P2. P1 is constitutively expressed and P2 is rpoE-dependent and induced under stress conditions known to induce the rpoE system such as misfolding of outer membrane proteins (5, 310, 330).

Regulation of *S.* Typhimurium σ^E is slightly different to that seen in *E. coli*. Three promoters have been identified: *rpoEp1*, *rpoEp2* and *rpoEp3*. These promoters were differentially expressed during growth and under stress conditions such as cold shock. *rpoEp3* is recognised by RNA polymerase holoenzyme containing σ^E. This promoter is induced by heat shock, osmotic stress and more strongly by cold shock and entry into stationary phase (256). Using this rpoE-dependent promoter, the previously established *E. coli* two plasmid system was optimised for the identification of promoters identified by *S.* Typhimurium σ^E (349). Further studies were involved in the characterisation of the σ^E –dependent *rpoEp3* promoter of *S.* Typhimurium which identified nucleotides critical for the *rpoEp3* promoter activity (255, 348). These studies also showed S. Typhimurium *rpoEp3* promoter activity to peak in early stationary phase and decrease in late stationary phase in contrast to the pattern of expression of σ^E activity seen in *E. coli*.

Unlike *E. coli*, *S.* Typhimurium *rpoE* was found to be non-essential when an *rpoE* knock out mutant was analysed (167) although it should be noted that rpoE is only non-essential under normal conditions and rpoE is essential to the survival of *S.* Typhimurium within the host. However, it was found to have a defective ability to utilize carbon sources other than glucose and showed increased sensitivity to hydrogen peroxide, superoxide and anti-microbial peptides. In addition, the *rpoE*

mutant showed a reduction in its ability to survive and proliferate in macrophage and epithelial cell lines. It was also shown that the *S.* Typhimurium *rpoE* mutant was attenuated in mice when infected by the intravenous route. As *htrA* has previously shown to be required for survival and virulence of *S.* Typhimurium, the above studies were repeated using the *htrA* mutant, to compare against results with the *rpoE* mutant. Results from this study showed that the *rpoE* mutant showed greater attenuation in mice and was unable to colonize in the liver and spleen of mice following IV infection. *htrA* mutants showed reduced growth in mouse tissue after both oral and IV infection, but were able to colonize the liver and spleen of mice to the higher levels seen in an *rpoE* mutant. These studies indicated the presence of further unidentified genes in the σ^E regulon of *S.* Typhimurium that could play a role in its virulence (167).

Recently colleagues within and outwith our laboratory have investigated the σ^E regulon of *S.* Typhimurium through microarray studies and identification of rpoE-dependent promoters (331, 349) and work is ongoing to characterise the role of these RpoE-regulated genes in *Salmonella* virulence, and also to investigate their potential as foreign antigen carrying attenuated vaccines.

Two *S.* Typhimurium σ^E regulated genes (*htrA* and *surA)* have been shown to be involved in virulence (167, 182, 364), and the interplay between these two genes and *skp* (a periplasmic chaperone) in *E. coli* has recently been defined (347).

fkpA, another *S.* Typhimurium σ^E regulated gene was recently studied to investigate its role in virulence (166). *fkpA* is a periplasmic peptidylprolyl isomerase (PPIase), which has a role in assisting protein folding by catalysing the isomerisation of prolyl residues in polypeptides (138). Results of this study showed *fkpA* to have only a minor effect on the virulence of *S.* Typhimurium though when combined with either

surA or *htrA*, making a double mutant, a much greater effect on virulence was observed (166).

As highlighted above, σ^E controls virulence functions in both *S.* Typhimurium and *E. coli* and this ESR response is necessary for protection against many environmental stresses such as reactive oxygen species (ROS), stationary phase survival, long-term carbon starvation survival, acidic pH, high temperature and polymyxin B (167, 190, 367).

The *P. aeruginosa* σ^E homologue known as AlgU, plays an important role in its pathogenesis however like *S.* Typhimurium this σ^E homologue is not essential (231). This is a bacterial pathogen of particular importance in cystic fibrosis sufferers, where it is the predominating pathogen, with 90% of sufferers eventually becoming colonized (107, 108). A phenotypic feature of *P. aeruginosa* strains that colonize CF patients is their ability to convert to a mucoidal, exopolysaccharide alginate over-producing form. This conversion to a mucoidal form promotes chronic bacterial infection with a poor clinical outcome (107).

Similarly to *S.* Typhimurium and *E. coli*, *P. aeruginosa* AlgU is thought to have a role in global gene expression (339) and the organisation of the AlgU operon, *algU mucA mucB mucC* is equivalent to the *rpoE rseA rseB rseC* operon structure of σ^E (228) with the additional *mucD* gene at the end of the AlgU operon which is a homologue of *htrA* (34). AlgU expression is controlled by two AlgU- dependent promoters upstream of *algU* (340). A recent study found 10 newly identified *P. aeruginosa* promoters that were dependent on the alternative sigma factor AlgU. Two genes identified as being controlled by AlgU are *lptA* and *lptB* that encode for putative lipoproteins. These lipoproteins were found to promote IL-8 production in primary human macrophages derived from peripheral blood monocytes (107).

Unfolded periplasmic proteins are a major inducing signal of the σ^E ESR with small RNA's playing a role in the control of porin expression and σ^E activity (373). The σ^E response down regulates expression of porins such as OmpA, OmpC and OmpW (187, 324) and this process is thought to involve two RpoE-dependent Hfq (an RNA chaperone)-binding sRNAs called MicA and RybB.

MicA and RybB are chromosomally encoded non-coding RNA's expressed in a growth phase dependent matter (12, 187, 381, 388). MicA was shown to be involved in the control of *ompA* through studies that showed overexpression of MicA led to a decrease in the cellular level of OmpA. It was discovered that MicA prevents ribosome binding by itself binding to the translation-initiation region of *ompA* mRNA. More recent studies have shown the transcription of MicA and RybB to be under the control of σ^E (106, 135, 181, 187, 382) and *ompC* and *ompW* mRNAs transcripts were shown to be destabilised by RybB expression. Similar to what is seen with *ompA*, rpoE-dependent down regulation of *ompC* and *ompW* is affected in cells that lack either RybB or Hfq.

1.5.3.2 CpxAR two-component signal transduction pathway

The Cpx A/R two-component signal transduction pathway is another ESR thought to be important to the virulence of many pathogenic bacteria. A model for regulation of the CpxRA pathway is shown in figure 1.4. It was first described in 1980 with the identification of Cpx mutants (238). The individual Cpx proteins were described and through their amino acid sequence, their precise role was identified (238, 358). CpxA is now known to be the membrane sensor that can sense extracytoplasmic stress,

while CpxR is the response regulator. The system acts like many two-component signal transduction pathways with a sensor kinase and a cognate response regulator that work together to sense and respond to changes within the cell. Further work has shown that various proteins such as MalE and NlpE (a new outer-membrane lipoprotein) could activate the Cpx signal transduction pathway through the CpxA sensor (63-65, 170, 350). Inducing signals of the Cpx system include increased expression of pili components (169) and overproduction of outer membrane proteins including NlpE (82, 259, 350) and its ultimate role is thought to be sensing and responding to proteins and other components that can accumulate and misfold in the periplasm.

Danese and Silvahy devised a screen to identify further Cpx regulated genes by looking for genes stimulated by the overproduction of NlpE, known to be regulated itself by CpxA/R. This screen identified the gene *cpxP* as being Cpx regulated. This gene encodes a periplasmic protein involved in combating extracytoplasmic protein medicated toxicity (64). CpxP is a protein that can bind to and inhibit cpxA activity in the absence of envelope stress. Several other genes have also been identified as Cpx regulated including *dsbA* (a periplasmic disulphide bond oxidoreductase that is utilised by both SPI1 and SPI2 TTSS) and *ppiA* (63, 299). LacZ transcription fusions and promoter mapping experiments found *dsbA* to be transcribed from yihE, a Cpx dependent promoter upstream of Cpx.

The Cpx system is thought to control the expression of many proteins, with at least 100 having been identified in *E. coli* (78) as Cpx regulated. The specific role of Cpx regulated genes can be sub-divided into three categories as follows:

Firstly, many of the Cpx regulated genes are involved in the protein folding and degradation within the protein envelope for example *htrA*, a chaperone and *ppiA*, a

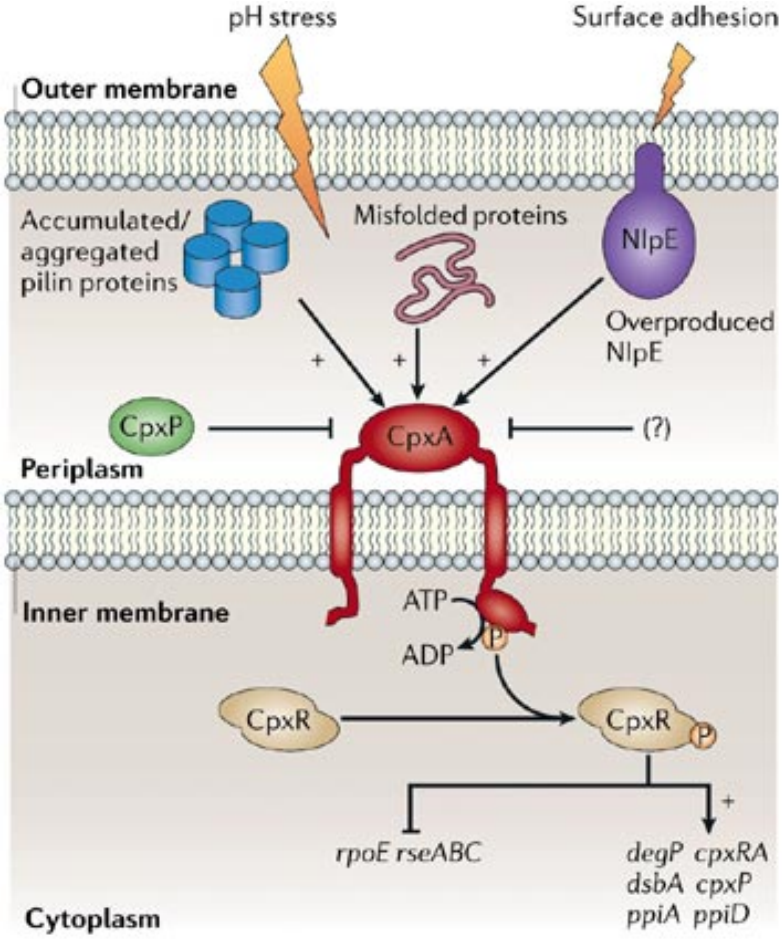


Figure 1.4. Model for regulation of the CpxRA pathway(332).

member of the peptidyl prolyl isomerase family, which are thought to be involved in the correct folding of proline-containing substrates (316).

Secondly, a further group of Cpx regulated genes are those that are involved directly in the signal transduction apparatus, such as *cpxP* which can inhibit *cpxA* in the absence of envelope stress (64).

A final class of Cpx regulated genes are those genes controlled by the Cpx machinery that have roles outwith envelope protein biogenesis. An example within this class is the *tsr* gene, which encodes a chemoreceptor and was identified as Cpx regulated based on the presence of CpxR binding sites within the promoter region of the gene (77, 360).

CpxA is a membrane sensor histidine kinase that can sense either pilus assembly or envelope protein misfolding caused by periplasmic stress. Upon sensing these misfolded proteins or pilus assembly, CpxA is activated and can then activate the response regulator CpxR via kinase/phosphatase activation (358). CpxR activation acts as the catalyst for the up regulation of expression of genes involved in protein folding or degradation of incorrectly folded proteins (316). In the absence of either pilus assembly or protein misfolding, CpxP, the periplasmic inhibitor protein, keeps cpxA in an inactive state.

The Cpx pathway is now also known to be involved in biofilm formation in *E.coli* (83, 84, 282) Biofilm formation is the surface attachment of bacteria to a solid surface through a matrix like substance (19) which can cause health issues due to increased antibiotic resistance, or economic issues through the effect of biofilms on certain surfaces (59). The involvement of Cpx in the formation of biofilms was further clarified through a study looking at the effect of biofilm lifestyle on gene expression in *E. coli* (19). Many genes in this study were up or down regulated in response to

biofilm formation, including many concerned with cellular processes including envelope stress response such as *cpxP*, Phage shock protein genes, *rpoE*, *rseA* and *rseB*. *cpxP* was one of the most over-expressed genes and mutation studies were performed to compare the difference in biofilm formation between strains with and without various members of the Cpx pathway. Results from this study showed a reduction in biofilm formation in mutants of the Cpx pathway, as well as *nlpE*, overproduction of which is a known inducer of the Cpx pathway. This study confirms the important role of the Cpx pathway in biofilm formation, and the importance of further study to fully elucidate the mechanism involved (84).

CpxA, R and P are known to be present not only in *E. coli* but also other pathogenic bacteria including *S.* Typhimurium (165, 190) *Yersinia* species (20, 159) and *Legionella pneumophila* (114). *S.* Typhi is known to share 100%, 100% and 88% homology with the respective proteins CpxA, R and P (76) of *E. coli* and this sequence similarity suggests that the Cpx pathway plays a similar role in *Salmonella* species.

Previous studies in *S.* Typhimurium have shown the Cpx system to play in role in the regulation of SPI-1 TTSS genes with CpxA being required for HilA expression at pH 6.0 (268). This ties in with recent work which has shown the Cpx pathway to be important in the *Y. pseudotuberculosis* type III secretion of *Yersinia* outer proteins (43).

Also, following the construction of Cpx mutants, it was discovered that a gain of function *CpxA** mutation (resulting in constitutive CpxA expression) caused a more extreme effect upon *S.* Typhimurium virulence than loss of either *cpxA* or *cpxR*. The *S.* Typhimurium *cpxA** also showed a reduced ability to colonise the Peyer's patches and peripheral organs of infected mice compared to a *cpxA* deletion mutant (165).

Other Cpx associated effects on *Salmonella* serovars include a reduced ability of *S.* Typhi to adhere to and invade epithelial cells *in vitro* when a Tn*pho*A insertion is present in *cpxA* (206).

1.5.3.3 Comparisons of the RpoE and CpxAR ESR systems

As can be seen from above, RpoE and CpxAR are both regulatory systems used by the bacteria to sense and respond to changes in the periplasmic proteins brought about through periplasmic stress.

Although these two systems share many similarities, there are several aspects of their machinery that differ.

σ^E is involved in alterations in outer membrane folding, whereas the Cpx pathway is involved in correcting misfolded envelope proteins at the periplasmic face, in particular the misfolded P pilus subunits.

The activating signal for the σ^E regulatory pathway is either misfolded outer membrane proteins or periplasmic proteins. In response to an initiating signal the interaction between the α sigma factor RseA and σ^E is lost, allowing σ^E to bind to RNA polymerase initiating up regulation of genes such as the envelope folding factor *fkpA*, the envelope protease *htrA*, members of the rpoE operon *rpoErseABC* and other virulence factors. Brought together, the σ^E regulatory pathway ensures proper outer membrane folding.

In contrast, the activating signal for the Cpx regulatory pathway is inner membrane associated aggregates of misfolded proteins. In response to this initiating signal, CpxA no longer binds to CpxP allowing phospho transfer from CpxA to CpxR.

Phosphorylated CpxR can then up regulate genes such as the envelope folding factors *dsbA, ppiA*, and *ppiD*, the envelope protease *htrA, cpxAR, cpxP* and other virulence factors. When activated, the Cpx pathway ensures appropriate pili biogenesis (315, 316).

Although the σ^E regulatory pathway and the Cpx two-component signal transduction pathways differ in many ways there are several elements of both systems that overlap with each other. Both pathways are known to be involved in HtrA expression, and both pathways have also been found to be involved in the formation of biofilm in *E.coli* (19, 315). In addition to playing a role in the expression of genes involved in protein folding, both pathways are also involved in the regulation of genes not concerned with protein folding. These other virulence-associated genes are downstream targets of both the σ^E and Cpx pathways. It is also known that the σ^E and Cpx pathways overlap with the σ^H cytoplasmic heat shock response mentioned earlier. The *P*3 promoter of rpoH is a target of σ^E (93, 387), while the Cpx pathway regulates expression of *ppiD*, which is also under the control of σ^H (63).

The σ^E and Cpx pathways can both auto-regulate their own expression. σ^E can activate its own expression and the expression of down stream negative regulatory genes *rseABC* that are all part of the σ^E operon (75, 310) and similarly Cpx can auto-regulate itself through the expression of genes *cpxA*, *cpxR* and *cpxP* (313).

Although these two pathways are known to overlap, the definitive reason for this has not been established. Similarly, both pathways appear to be involved in the pathogenesis of the organism, though the precise mechanisms for signal detection and their physiological role are not yet fully understood.

As such, there is still much to be learned about these stress response pathways and how they interact together.

1.5.3.4 BaeSR

A third envelope stress signal transduction pathway in *E. coli* (309), known as BaeSR has been identified. It is another example of a two-component regulatory system, whereby the BaeS is the sensor kinase and BaeR is the response regulator. BaeSR is able to control the expression of *Spy* in response to envelope stress. *Spy* is a periplasmic protein whose expression is induced by spheroplast formation and which was found to be under the control of the cpx pathway (139, 312). *Spy* shares limited homology with CpxP, though it does not function in the same way and certain inducing signals of the Cpx system (spheroplasting and PapG overexpression) are also involved in activating the Bae system.

The number of genes thought to be regulated by BaeSR has expanded following a microarray study which looked at overexpression of BaeR. This identified 59 genes that were activated in response to BaeR overproduction, including genes involved in flagellar biosynthesis and chemotactic responses (272). Interestingly, this microarray study identified genes *ibpA* and *ibpB* as being repressed following BaeR overproduction. The genes *ibpAB* are known to be up-regulated in *S.* Typhimurium following rpoE overexpression and are studied further in this thesis. This potential co-regulation of *ibpAB* by BaeSR and RpoE, as well as the co-regulation of *spy* by BaeSR and CpxAR highlights how the ESR systems appear to interact with each other. Other work has shown overexpression of BaeR to increase resistance to compounds such as antibiotics and bile acids (15, 159-161) which has been supported by a study showing *baeSR* deletion mutants to play a role in the control of antimicrobial resistance systems (407). Hopefully, further studies can expand on the

possible function of this system in the ESR of *E. coli* and potentially other pathogenic bacteria.

1.5.3.5 Phage Shock Protein Response

The phage shock protein response (psp) has recently been regarded as a fourth ESR system and is studied in more detail in chapter four of this thesis.

It can be induced in *E. coli* following certain signals such as extremely high temperature, hyperosmotic shock, ethanol exposure (38, 196, 261), stationary phase survival (3), CCCP exposure (17) and lipid biosynthesis inhibition (22).

At present this system is known to be present in *E. coli*, *S.* Typhimurium and *Y. enterocolitica* and further study should yield more information on its regulon members, physiological functions, interaction with other ESR systems and possible presence of other pathogenic bacteria.

1.6 *Salmonella* Therapeutics

A recent *Salmonella* conference (ASM, Canada, 2006) brought together current thoughts and latest findings under the broad title *Salmonella*: from pathogenesis to therapeutics (36).

As mentioned previously, there are 20 million cases and 200,000 deaths associated with Typhoidal *Salmonella* serovars (61). It is wide-spread in Southeast Asia, Africa and South America with high incidences of recurrent infection (288) and it is complicated further by the emergence of antibiotic resistance. In particular there has

been a significant increase in the resistance to the antibiotic nalidixic acid, which is commonly used in the treatment of disease caused by Typhoidal *Salmonella* (343).

The clinical outcome for patients diagnosed with antibiotic-resistant Typhoidal *Salmonella* infection is poor, exacerbated by the belief that serovars acquiring increased drug resistance may develop an increased virulence potential, resulting in greater mortality (130).

The emergence of multi-drug resistant nontyphoidal Salmonellosis is also of concern, in part due to its economic impact but also due to the clinical complications that can arise in immunocompromised and at-risk patients. For example, multi-drug resistant nontyphoidal Salmonellosis is the most common cause of bacteraemia in children under 5 in African countries (130). These figures highlight the need for new therapeutics targeted against *Salmonella* infection.

In Vietnam there is a group currently involved in the development of methods to test antimicrobials against *Salmonella* serovars, with the aim of establishing local clinical drug trials for quick, efficient analysis of these antimicrobials whilst also providing an accessible source of information on the efficacies of such drugs. These clinical trials are presently involved in the study of two new-generation antibiotics, azithromycin and gatifloxacin, effective against drug-resistant *Salmonella* (287).

In addition to studies mentioned above, there is much research on-going into new therapeutic approaches against *Salmonella* infection that take advantage of various aspects of *Salmonella* pathogenicity. Several groups, for example are involved in utilising specialised virulence associated organelles such as the TTSS as therapeutic drug targets (127, 274). It is thought that targeting drugs aimed at inhibiting the function of such organelles would affect the pathogenicity of the bacteria involved, rather than its survival and as such should reduce the likelihood of resistance

developing. Also, as these specialised virulence organelles are usually absent in non-pathogenic bacteria there is thought to be reduced prospects of an alteration in the host normal bacterial flora.

Working along a similar theme, are groups involved in targeting the function of pathogenic virulence factors such as YopH in *Y. pestis* and inhibiting their action (162, 215). Such strategies involved in utilising the interface between *Salmonella* and the host are in the very early stages, and it remains to be seen how effective these approaches are (289).

Given the vast impact that *Salmonella* infection has on global health and economy, it is crucial to investigate the development of new efficacious vaccines.

Parenteral Vi polysaccharide and oral Ty21a typhoid vaccines are currently those recommended in the control of endemic Typhoid (212). The Vi polysaccharide is an injection based vaccine and as such not ideal in developing countries where *S.* Typhi predominates due to issues such as lack of refrigeration equipment and cross-contamination of needles. The oral Ty21a vaccine is a live attenuated vaccine, and its avirulence was once thought to be due to a mutation in *galE,* a gene in the galactosidase utilization pathway that affects O-antigen production. Varying efficacy as low as 60 to 80% in Chile and concern that the strain may not survive long enough within the Peyer's patches to mount a strong immune response due to the unknown nature of the mutation in the strain have led to a need for a safer, more efficient oral, live vaccine. In addition, there is concern at the increase in the development of paratyphoid fever caused by *S.* Paratyphi A and B (276) as there is no vaccine licensed at present against these *Salmonella* serovars. The current polysaccharide Vi vaccine cannot protect against *S.* Paratyphi A or B as neither of these two *S.* Paratyphi serovars contain the Vi antigen that is responsible for protection against *S.* Typhi. A

recent article has investigated the potential for the currently available Ty21a typhoid vaccine to prevent paratyphoid fever which found cross- protection was observed in 58% of cases caused by serovar Paratyphi B, while there was no protection found against serovar Paratyphi A (36, 211).

The current strategy for developing a live oral vaccine is to take avirulent mutant strains of *S.* Typhimurium or *S.* Typhi with known attenuation and introduce genes encoding various antigens into the strain (317, 326, 327, 364) with the bacteria expressing the desired antigen on their surfaces. *Salmonella* is a good candidate as it multiplies in Peyers patch, and stimulates good humoral and mucosal response. There are obvious benefits to designing an *S.* Typhi vaccine carrying antigen gene from another bacteria to confer effective protection against multiple pathogens.

1.7 Aims of thesis

Our lab is concerned, amongst other things, with investigation of the pathways controlled by the alternative sigma factor σ^E, the cpxAR two-component regulator and the psp ESR systems in *S.* Typhimurium.

Aims of this thesis were firstly to characterise further, specific genes presumed to be members of the *S.* Typhimurium σ^E regulon following analysis of microarray data collated previously by colleagues within our laboratory (331). Clarification of rpoE-dependency was achieved with the use of quantitative RT-PCR (QRT-PCR). Mutated versions of these genes were constructed and subsequently characterised both *in vitro* and *in vivo*, with the ultimate aim of identifying possible vaccine candidate genes.

Following this the fourth ESR, the psp response of *S.* Typhimurium, was characterised more fully by creation of deletion mutations and regulation studies followed by *in vitro* and *in vivo* studies to help clarify the role this ESR plays in *S.* Typhimurium infection.

Microarray studies were established to identify members of the CpxAR regulon of *S.* Typhimurium in concert with QRT-PCR and characterisation of genes which may be co-regulated by both the σ^E and CpxAR ESR systems was also performed.

Preliminary vaccine studies were set-up to investigate the potential of genes which I identified as possible vaccine targets and final studies involved the analysis of the structural components of htrA, an important stress response protein known to be regulated by both σ^E and CpxAR that is also known to be crucial to the pathogenesis of *S.* Typhimurium.

In summation, the aims of this thesis were to characterise the ESR systems of S. Typhimurium to widen our current knowledge of genes involved in these systems and their role in the pathogenesis of *S.* Typhimurium with the ultimate aim of identifying possible candidate vaccine genes that may be used in future therapeutics against *Salmonella* infection.

Chapter Two – Materials and Methods

2.1 Bacterial Strains and Plasmids

Bacterial strains and plasmids used and constructed during this study are shown in tables two and three

Table two – A list of Bacterial strains used or constructed throughout this study

Name	Genotype	Source
SL1344	*S.* Typhimurium *his* mutant, mouse virulent strain	Roberts lab
Sty12023	mouse virulent strain	Roberts lab
GVB1343	SL1344 htrAΔ ::Kan	Roberts lab
GVB1335	SL1344 pKD46	
GVB1327	Sty12023 pKD46	
GVB311	SL1344 *rpoE*::Kan	Roberts lab
GVB368	SL1344 *cpxR*::Kan	Roberts lab
GVB822	SL1344 *cpxA*::Gm	Roberts lab
GVB870	SL1344 *cpxA**	Roberts lab
SMS348	SL1344 *rpoS*::Amp	
GVB852	SL1344 *rpoN*::tet	SGSC
BRD1115	C5 Δ*surA*	(364)
GVB387	SL1344 *fkpA*::kan	Roberts lab
BRD915	SL1344 Δ*htrA*	(49)
GVB1360	SL1344 *ΔtolR*	Roberts lab
GVB1373	PSP region in Top 10 cells : amp	This study
GVB1371	SL1344 *Δpsp*: Kan	This study

Name	Genotype	Source
GVB1715	12023 *ΔpspC*: Kan	This study
GVB1716	SL1344 *ΔpspC*: Kan	This study
GVB1736	12023 *ΔslyB*: Kan	This study
GVB1737	SL1344 *ΔslyB*: Kan	This study
GVB1738	120223 *ΔnlpB*: Kan	This study
GVB1739	SL1344 *ΔnlpB*: Kan	This study
GVB1740	12023 *ΔsmpA*: Kan	This study
GVB1741	SL1344 *ΔsmpA*: Kan	This study
GVB1742	12023 *ΔibpA*: Kan	This study
GVB1760	12023 *ΔibpB*: Kan	This study
GVB1776	SL1344 *ΔibpB*: Kan	This study
GVB 1761	12023 *ΔygfM*: Kan	This study
GVB1777	SL1344 *ΔyfgM*: Kan	This study
GVB1762	12023 *ΔychH*: Kan	This study
GVB1764	SL1344 *ΔychH*: Kan	This study
GVB1763	12023 *ΔpqiA*: Kan	This study
GVB1765	SL1344 *ΔpqiA*: Kan	This study
GVB1781	12023 *Δddg*: Kan	This study
GVB1795	SL1344 *Δddg*: Kan	This study
GVB1802	12023 *ΔpspA*: Kan	This study
GVB1806	SL1344 *ΔpspA*: Kan	This study
GVB1807	12023 *ΔibpAB*: Kan	This study
GVB1808	SL1344 *ΔibpAB*: Kan	This study

Name	Genotype	Source
GVB1829	12023 *ΔpspA*: Chlor	This study
GVB1831	SL1344 *ΔpspA*: Chlor	This study
GVB1833	SL1344 *ΔpspA ΔrpoN* : Chlor, Kan	This study
GVB1834	SL1344 *ΔibpA*: Kan	This study
GVB1836	SL1344 *ΔfliC*: Kan	This study
GVB1838	SL1344 *ΔfljB*: Kan	This study
GVB1839	SL1344 *ΔtolB*: Kan	This study
GVB1840	SL1344 *ΔtolBpalybgF*: Kan	This study
GVB1845	SL1344 *ΔtolQRA*: Kan	This study
GVB1848	SL1344 *ΔfliC ΔfljB*: Kan, Gent	This study
GVB1851	SL1344 *Δpsp ΔpspG*: Kan, Chlor	This study

Table three – A list of plasmids used or constructed throughout this study

Name	Features	Source
pKD46	pINT-ts derivative containing araC-P_{araB} and $\gamma\ \beta\ exo$ DNA fragments, Ap^R	(72)
pKD4	pANT-Sγ derivative containing an FRT-flanked Kan^R gene, Ap^R	(72)
pKD3	pANT-Sγ derivative containing an FRT-flanked Cm^R gene, Ap^R	(72)
pCP20	bla cat cI857 lPR flp pSC101 oriTS	
pTL61t	Low copy, promotorless *lacZ* fusion vector Ap^R	
PCR 2.1	PCR topisomerase cloning vector	Invitrogen
pAC7	Low copy P_{BAD} expression vector, Cm^R	(322)
pAC-rpoEST4	PAC7 containing *rpoE* under control of the P_{BAD} promoter	(256)

Name	Features	Source
pWSK29	Ap^r; low copy number vector P_{BAD}	
pPSPF	Ap^R; pspF promoter region in pTL61t	This study
pPSPA	Ap^R; psp promoter region in pTL61t	This study
pCS20	*E. coli htrA* in pQE60	(355)
pCS21	*E. coli htrA* variant in pQE60	(355)
pIC1	*E. coli htrA* variant in pQE60	(355)
pIW5	*E. coli htrA* variant in pQE60	(355)
pIW7	*E. coli htrA* variant in pQE60	(355)
phtrA1	pCS20 under control of salmonella *htrA* promoter	This study
phtrA2	pCS21 under control of salmonella *htrA* promoter	This study
phtrA5	pIC1 under control of salmonella *htrA* promoter	This study
phtrA4	pIW5 under control of salmonella *htrA* promoter	This study
phtrA3	pIW7 under control of salmonella *htrA* promoter	This study
pND18	*nlpE* under the control of P_{BAD}	(65)

2.2 Bacterial media and routine growth conditions

All strains used in this study, were routinely cultured in Luria-Bertani broth (LB) (Oxoid Ltd, Basingstoke, Hampshire, UK) or on LB agar (LA) (LB broth supplemented with 1% (w/v) bacteriological agar, No.1, Oxoid Ltd), unless specified. Media was sterilised in an autoclave for 15 minutes at 121^{o}C and where required, supplemented with antibiotics at the following concentrations: 100µg/ml ampicillin (Sigma), 75µg/ml kanamycin (Invitrogen), 100µg/ml of streptomycin (Sigma), 30µg/ml chloramphenicol and 12.5µg/ml tetracycline. Antibiotics were resuspended

in distilled water except for tetracycline which was resuspended in 50% (v/v) ethanol. Agar plates were incubated statically overnight at 37^{o}C while 5ml overnight cultures used for subculture were grown with aeration at 37^{o}C in a rotary shaker (Stuart SI50), unless otherwise specified.

For SPI-1 inducing conditions cultures were grown in 3 ml LB broth for 37ºC, 225 rpm for 16 hours. A 1 ml sample of each culture was washed twice in sterile phosphate-buffered saline (PBS), re-inoculated 1:50 (v/v) into 10 ml LB and grown for 3 hours at 37ºC, 225 rpm to mid-log phase.

For SPI-2 inducing conditions cultures were grown in 11 ml Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 1% glutamine (v/v) at 37ºC, 5% CO_2/95% air (v/v) for 16 hours. Cultures were re-inoculated 1:50 (v/v) into 500 ml DMEM supplemented with 1% glutamine (v/v) and grown for 7 hours at 37ºC, 5% CO_2/95% air (v/v) to mid-log phase.

Pure cultures of all strains constructed were stored on microbank beads (ProLab) and kept at -80^{o}C. When culturing from freezer stocks, 1 bead was removed and streaked onto a fresh LA plate containing the appropriate antibiotic.

2.3 Recombinant DNA techniques

2.3.1 Isolation of Bacterial Chromosomal DNA

To prepare chromosomal DNA for use in polymerase chain reactions (PCR), a boilate method was followed. A single colony was picked from a fresh plate culture and resuspended in 200µl sterile dH_2O. After boiling for 5 minutes at 100^{o}C, the

suspension was centrifuged at 5000rpm in a microcentrifuge (Eppendorf 5415 D). 2µl of the neat supernatant was used in a 50µl PCR reaction.

2.3.2 Isolation of Plasmid DNA

A QIAPrep miniprep kit (Qiagen) was used for isolation of pure plasmid DNA for several applications, including use as template DNA for PCR amplification of plasmid specific genes to be used in Red mutagenesis, as a cloning insert or electroporations. Methods were followed as shown in the QIAprep Miniprep protocol for use with microcentrifuges. Briefly, 3ml of pelleted bacterial cells were resuspended and lysed using the buffers provided before being centrifuged for 10 minutes at full speed. The supernatant was then decanted to a QIAprep column and centrifuged for 1 minute. Flow through from this was discarded and following a final wash in buffer, plasmid DNA was eluted from the column using 50µl dH_2O.

2.3.3 QIAquick PCR purification

For direct purification of PCR products from amplification reactions and DNA clean up from other enzymatic reactions, a QIAquick PCR purification kit (Qiagen) was used. Methods were followed as shown in the QIAprep Miniprep protocol for use with microcentrifuges. Briefly, 5 volumes of a Qiagen buffer was mixed with the PCR sample (or DNA) and placed in a QIAquick spin column. To bind the DNA, the column was spun in a microcentrifuge for 1 minute. The flow through was discarded and the column was washed in another Qiagen buffer and spun for 1 minute. Flow through was again discarded and the column was spun for 1 minute. The QIAquick

column was then placed in a clean microcentrifuge tube and the DNA was eluted from the column using 50µl dH_2O (unless otherwise specified).

2.3.4 Pellet Paint ethanol precipitation

Pellet paint co-precipitant NF (Novagen) is a non-fluorescent carrier which was used in ethanol precipitation reactions to allow for easier DNA visualisation. This procedure was used on DNA following restriction endonuclease digestion. 1µl of Pellet paint was added to the DNA sample, followed by 0.1 volume of 3M Na Acetate and 2 volumes of absolute ethanol. The sample was than vortexed briefly and incubated at room temperature for 2 minutes. Following a 5 minute spin in a microcentrifuge at top speed, a coloured pellet was visible at the bottom of the tube. The supernatant was removed, and the pellet was then washed in 70% ethanol and 100% ethanol, respectively, followed by a 5 minute spin at top speed after each washing step. The supernatant was then removed and the pellet allowed to air dry. The dry pellet was then resuspended in 10µl of dH_2O, or at a concentration of 2µg/µl.

2.3.5 Electroporation of Bacterial Cells

For routine transformation of plasmid DNA, electrocompetent cells were prepared. 5ml overnight cultures of the appropriate strain were cultured in LB broth at 37^{o}C. The overnight was then diluted 1/100 into 50mls of fresh LB broth, and grown for around 3 hours until mid logarithmic phase ~0.6 OD600nm. (Beckman DU640 spectrophotometer). Cells were harvested in a bench top centrifuge (Jouan CR3) at 5000rpm for 10 minutes at 4^{o}C. The bacterial pellet was resuspended in 30mls ice

cold dH_2O and centrifuged as above. Cells were washed a further two times in ice cold dH_2O followed by a final wash in ice-cold 10% glycerol to remove any excess salts from the growth media. Between all centrifugations the cells were stored on ice. After washing, the final pellet was resuspended in 500µl of ice cold 10% glycerol (i.e.) one hundredth of the initial starting inoculum. Cells were stored on ice or at -80^{o}C until required for electroporation. 3µl of plasmid DNA was added to 50µl of prepared cells and left on ice for 30 minutes. The mixture was transferred to a chilled electroporation cuvette with a 2mm path length (Equibio) and electroporated on a BioRad Gene Pulser II electroporator using a resistance of 600Ohms, 25uF capacitance and 1.75 volts for both *E. coli* and *S.* Typhimurium. Immediately following pulse, 1ml salt optimised broth with carbon (SOC) media (0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$, 20mM glucose) (335) was added to the bacteria which were allowed to recover at 37^{o}C for 1 hour.100µl aliquots of cells were plated out on LA plates containing the appropriate antibiotic for selection. A "no DNA" negative control was set up for each experiment.

2.3.6 Transformation Using Chemically Competent Commercial *E. coli* Cells

For some ligation reactions and for cloning using the PCR2.1 kit described in the DNA manipulation section, chemically competent *E. coli* TOP10' cells (Invitrogen) were utilised. An aliquot of DNA was added to the one shot cells and left on ice for 30 minutes, after which they were temperature shocked at 42^{o}C for 30 seconds. Immediately after shock, 250µls of SOC were added to the cells which were then

allowed to recover at 37°C for 1 hour. As with electroporation the transformed cells were plated out on LA agar plates with the appropriate selection.

2.3.7 P22 Phage mediated Transduction of *S.* Typhimurium.

P22 transduction was used to transfer gene mutations marked with antibiotic selection markers between strains of *S.* Typhimurium, typically from the Sty12023 strain to the SL1344 strain. This procedure was performed using the bacteriophage P22 and the method was adapted from that described by (245). Dilutions of the P22 stock were made in LBEDO broth (LB containing 2% 50 X E salts and 1% glucose), neat through to $1x10^{-6}$, 10µl of which were mixed with 100µl of a 5ml overnight culture containing the genetic loci to be moved. This mix was incubated at 37°C for 30 minutes and then added to 3ml of top agar (LB broth containing 0.7% agarose), maintained at 42°C, and then poured over the surface of a dry LA plate containing appropriate antibiotics. After incubation for approximately 6 hours, or until plaques were visible, plaques were harvested from the plate which was just confluent. This was done by scraping off the top agar into a 50ml centrifuge tube with the addition of 3mls L-BEDO and 4 drops of chloroform from a pastette, and incubating at 37°C for 30 minutes with shaking. This mixture was then centrifuged at 5000rpm for 10 minutes, the supernatant from which was removed and filtered through a 0.2µm filter syringe. More chloroform (4 pastette drops) was added to this P22 lysate which can then be stored at 4°C. 100µl dilutions of this phage are added to 200µl of a 5ml overnight of the strain to be transduced and incubated at 37°C. After 30 minutes 1ml LB broth containing 10mM EGTA was added and incubated for a further 60 minutes. At this point 100µl was plated onto LA plates containing 10mM EGTA and the selective

antibiotic. Colonies recovered were checked by PCR to confirm that they contained the appropriate strain.

2.4 Molecular Cloning Techniques

2.4.1 Topisomerase Cloning

This method utilised the PCR2.1 kit (Invitrogen) which allows direct cloning of a *taq* polymerase PCR product (poly A tailed proof read PCR product) into cloning vector PCR2.1, via a topisomerase reaction which is covalently bound to the vector. PCR2.1 uses blue/white screening on LA plates containing 50μg/ml of 5-bromo-4-chloro-3-indolyl-B-galactopyranoside to identify the correct insertion, with white colonies containing the desired insertion due to disruption of the vector *lacZ* gene.

2.4.2 Restriction Endonuclease Digestion of DNA

Restriction endonuclease reactions were used for several cloning experiments, and also in the methodology used to create mutant strains using the RED recombinase procedure. Restriction endonucleases were supplied by Invitrogen except for *dpnI* which was supplied by Promega. DNA digests were set up in a standard digest mixture composed of 1x the appropriate restriction digest buffer, up to 2μg of DNA, 5-10 units of each restriction enzyme, and sterile dH_2O up to a volume of 25μl. The reaction was incubated in a 37^{o}C water bath (Grant) for at least 3 hours.

2.4.3 DNA ligation

For the production of template DNA used in cloning experiments and the RED recombinase experiments, DNA product was made by PCR amplification using oligonucleotides incorporating desired restriction sites into the 5' and 3' of the resulting product. The PCR product was then purified using a Qiaquick PCR purification kit (Qiagen) and checked for size on an agarose gel. For ligation reactions, an ethanol precipitation procedure was used on both vector and insert DNA after digestion using pellet paint (Novagen). The resulting pellets were resuspended in 10µl of dH2O and 1µl observed by agarose gel electrophoresis. The ligation reaction containing 1x ligase buffer and 1 unit of T4 DNA ligase (Invitrogen) usually consisted of a 1:5 vector: insert ratio, with approximately 10-50ng of vector per reaction. The ligase reaction was made up to 20µl with dH_2O and incubated overnight at 14^{o}C. A negative control ligation reaction was included for each digested vector used.

2.4.4 Screening of Recombinants

Putatively positive clones either from topisomerase cloning or traditional ligase reactions determined by white/blue selection or selective antibiotic resistance were screened for inserts. Typically this was by restriction digestion or where difficult by PCR amplification purified mini-prep plasmid DNA.

2.4.5 Agarose Gel Electrophoresis

DNA digest fragments and PCR products were examined by TBE-agarose gel electrophoresis of various concentrations between 1 and 2% depending on DNA size, upon addition of a sample loading dye. Gels were typically run at 100V for up to 1 hour. DNA was visualised on a transilluminator through addition of 10µg/ml Ethidium bromide to the agarose. DNA quantification and size were estimated through comparison with Hyperladder I and Hyperladder IV DNA standard markers (Bioline).

2.4.6 Polymerase Chain Reaction

All PCR reactions were performed on a Hybaid PCR express machine. For PCR screening a *taq* master mix (Bioline) containing 2.5mM $MgCl_2$, was used. A typical 50µl reaction contains 25µl of the master mix, 2µl of 5' and 3' oligonucleotides, 2µl of template DNA and 19µl dH_2O. For amplification of PCR products destined for subsequent cloning events where proof reading is required, a similar master mix, bio-XACT (Bioline) was used which contains a proof reading polymerase. Standard PCR reactions consisted of an initial denaturation at 95^oC; 2mins followed by 30 cycles of 94^oC; 40secs, anneal; 30secs and a 72^oC extension, with a final extension period of 72^oC; 10mins. The annealing temperature for each primer set was optimised and for all primers was between 52 ^oC and 56 ^oC. Oligonucleotides for the purpose of generating insert for cloning and for screening were synthesised by MWG Biotech

whilst oligonucleotides designed for RED mutagenesis were synthesised by Sigma. All oligonucleotides used in this study are listed in table four.

2.5. β-galactosidase Assays

β-galactosidase assays were performed on *S.* Typhimurium cultures transformed with *lacZ*-promoter fusion reporter plasmids. The assay used is based on that of Miller (245), but adapted into a 96 well micro titre plate format (133). 5ml overnight cultures were grown with ampicillin to select for the reporter plasmid, and diluted 1/100 into 5ml fresh LB broth. Cells were grown at 37ºC until they reached an OD 600nm measurement of approximately 0.6 and 1.0 respectively. These time points were chosen to represent cells at both mid-log phase, and at stationary phase. At each time point, 1ml of cells was removed for spectrophotometer reading, following which 70µl of these cells were placed into an eppindorf tube. To lyse the bacterial cells, 1 drop of chloroform and 1 drop of 1% SDS were added and each tube was then vortexed for 10 seconds. 10µl of the above mix was added to 6 wells on a 96 well plate containing 90µl of β- galactosidase assay buffer per well (60mM Na2HPO4.7H20, 40mM NaH2PO4.H20, 10mM KCl, 1mM MgSO4.7H2O, 50mM β- mercaptoethanol). The reaction was started with 25µl of ONPG (O-nitro phenyl galactosidase, Sigma) which was prepared in the above assay buffer without the addition of β- mercaptoethanol. The reaction was stopped with 50µl 1M Na2CO3 when the wells had proceeded to a dark yellow colour. When both time points had been completed, the plates were read at an OD of 420nm. Promoter activity of the respective cells were measured in Miller units and calculated as shown below.

Miller units =

$$\frac{(1000 \times OD420)}{\text{Volume of cells (mls)} \times OD600 \times \text{Time (mins)}}$$

The above β-galactosidase assay was adapted in certain experiments to allow arabinose induction of the pBAD promoter as follows:

For arabinose induction of strains containing the pBAD promoter, overnight cultures were diluted 1:1000 into LB broth plus 0.2% glucose, with the appropriate antibiotics and allowed to grow to early log phase (taken to be an optical density at 600nm of approximately 0.2).

Bacteria were then collected by centrifugation and diluted to an OD600 of approximately 0.03 to 0.05 in pre-warmed LB broth plus 0.2% glucose (for the non-inducing control) or 0.2% arabinose (for inducing cultures). Samples were taken at various time points and assayed for β-galactosidase activity. All experiments were repeated in triplicate and data from a representative experiment was plotted.

2.6.1 Protein extraction of SPI-1 and SPI-2 expressed proteins

S. Typhimurium strains were grown under SPI-1-inducing conditions to an optical density of 0.6 at 600 nm. OD_{600} values of each culture were standardised to normalise protein volumes in each. Bacterial cells were removed from the cultures by centrifugation at 5,000 rpm for 15 min, followed by filtration of the supernatant through a 0.2 μm-pore size low protein binding filter. Proteins from the culture supernatant were precipitated by addition of 10% (v/v) cold trichloroacetic acid, followed by incubation at -20ºC for 15 min, then at

4ºC for 30 min. Precipitated proteins were recovered by centrifugation at 5,000 rpm for 45 min. Pellets were resuspended in 10 ml ice-cold ethanol, held on ice for 15 min and centrifuged at 5,000 rpm for 15 min. Pellets were resuspended in 1 ml ice-cold ethanol, held on ice for 15 min and centrifuged at 16,000 rpm for 5 min. Once dried, pellets were resuspended in 100 µl Laemelli's Sample buffer 9 and boiled at 95ºC for 15 min to allow protein denaturation. Bacterial whole cell (WC) fraction pellets were washed twice in sterile PBS and optical density values at 600 nm were normalised prior to centrifugation at 16,000 rpm for 5 min. Once dried, pellets were resuspended in 50 µl Laemelli's Sample buffer 9 and boiled at 95ºC for 15 min. When necessary, samples were neutralised by addition of 2 µl Tris HCl prior to storage at -20^{o}C.

S. Typhimurium strains were grown under SPI-2 -inducing conditions in DMEM to an optical density of 0.6 at 600 nm. An excel package was used to standardise the OD_{600} values of each culture to normalise protein volumes in each. Bacterial cells were removed from the cultures by centrifugation at 10,000 × *g* for 15 min, followed by filtration of the supernatant through a 0.22 µm-pore low-affinity protein binding pump filter. Filtered cell-free culture supernatants were stored at -80ºC for 24 hours prior to lyophilisation. Lyophilised cultures were centrifuged at 10,000 x *g* to remove salts. Proteins were precipitated by addition of 10% (v/v) cold trichloroacetic acid, followed by incubation at -20ºC for 15 min, then at 4ºC for 30 min. Precipitated proteins were recovered by centrifugation at 10,000 × *g* for 45 min. Pellets were resuspended in 20 ml ice-cold ethanol, held on ice for 15 min and centrifuged at 5,000 rpm for 15 min. Pellets were resuspended in 1 ml ice-cold ethanol, held on ice for 15 min and centrifuged at 16,000 rpm for 5 min. Once dried, pellets were resuspended in 100 µl Laemelli's Sample buffer 9 and boiled at 95ºC for 15 min to allow protein denaturation. Bacterial whole cell (WC) fraction pellets were washed twice in sterile PBS and optical density values at 600 nm were

normalised prior to centrifugation at 16,000 rpm for 5 min. Once dried, pellets were resuspended in 100 µl Laemelli's Sample buffer 9 and boiled at 95ºC for 15 min. When necessary, samples were neutralised by addition of 2 µl Tris HCl prior to storage at -20^{o}C.

2.6.2 SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the standard method described by Laemmli (202). For analysis of protein samples, ready made 12% acrylamide gels were used (BioRad). Whole cell protein samples were prepared from 5ml overnight bacterial cultures adjusted to ensure consistent loading between lanes and strains. Aliquots were centrifuged at 15,000g in a microcentrifuge, resuspended in 100µl Laemmli sample buffer (Sigma), and boiled for 5 minutes at 100^{o}C for 5 minutes prior to loading. For size comparison a precision plus protein standard marker (BioRad) was used. SDS-PAGE gels were run at 200V for 45 minutes.

For Coomassie Staining, gels were incubated at room temperature for 18 hours in Coomassie Brilliant Blue stain, with rocking. Gels were then de-stained appropriately. For western blot analysis, SDS-PAGE proteins were transferred to nitrocellulose membrane in a 1.44% glycine, 0.3% Tris, 20% methanol transfer buffer, using BioRad western blotting apparatus for 1 hour at 100V. The nitrocellulose paper was then blocked in a 10% marvel PBS-0.05% Tween 20 solution for 30 minutes. The primary antibody, usually at 1/1000 dilution, was added in 5% Marvel PBS-Tween and left to shake on an orbital shaker for at least 2 hours. Unbound antibody was

removed by washing three times with PBS-Tween, for 15 minutes each. An anti-species secondary antibody conjugated to horse radish peroxidase (HRP) was diluted 1/1000 in 5% marvel PBS-Tween and added to the nitrocellulose for at least 1 hour. Prior to developing, the blot was washed 3 times in PBS-Tween and twice in PBS. HRP substrate solution (30mg chloronapthol in 20ml methanol, 80ml PBS and 100ul 30% H_20_2) was added to the blot and left until colour developed and protein bands visualised.

2.7 Construction of *S.* Typhimurium mutants using the λ red recombinase system

S. Typhimurium mutants were constructed using an adapted method of the λ red recombinase system described previously by Datsenko and Wanner (72, 331)

2.7.1 DNA preparation

Bulk PCR samples were set up using either pKD3 or pKD4 as the template plasmid depending on which antibiotic resistance cassette was required. To provide a final volume of 1.2ml PCR product, 12 x 100µl PCR reactions were pooled together following amplification. Oligonucleotides (Sigma Genosys) were designed with 20bp homology to the antibiotic cassette, and 40bp homology to the chromosomal loci of interest. All oligonucleotides used in the construction of *S.* Typhimurium mutants are shown in table four. Due to the volume of the PCR reactions required a *taq* polymerase master mix was used as described in 2.4.6, with the following cycle

conditions, 94°C;5 minutes followed by 30 cycles of 94°C;60secs, 55°C;60secs, 72°C;90secs and then a final extension period of 72°C;10mins.

Following amplification, the pooled PCR products were cleaned up by QIAquick PCR purification (Qiagen), as described in 2.3.3. Following purification, the DNA was digested using *dpnI* to remove the template plasmid (as described in 2.4.2) re-cleaned using the QIAquick PCR purification kit, and eluted with 100µl of ddH_2O.

2.7.2 Lambda Red Swap

S. Typhimurium mutants were constructed using an adapted protocol of the λ red recombinase system described previously by Datsenko and Wanner (72, 331) as shown.

SL1344 pKD46 or Sty12023 pKD46 electrocompetent cells carrying the temperature sensitive helper plasmid pKD46 were prepared following overnight growth of 5ml cultures at 30°C in LB-Amp. Cells were diluted 1/100 into 100mls fresh LB-Amp carrying 1mM L-arabinose, and grown at 30°C with shaking until they reached an A600 absorbance of 0.6 (approximately 3 to 4 hours). Cells were concentrated 100 fold to provide 10 x 100µl aliquots. 100µl of electrocompetent cells were mixed with 0, 1, 5, 10, 25, or 50µls of purified DNA, and rested on ice for 30 minutes. After electroporation, cells were recovered for 1 hour at 37°C, as shown in 2.3.5. 100µl of cells were then plated out on LA plates carrying the appropriate antibiotic and incubated at 37°C overnight. In samples where no colonies were seen, the remaining 900µl of cells were stored at room temperature overnight, centrifuged at 5000rpm for 2 minutes, and then resuspended in 100µl SOC before being plated out on LA plates

carrying the appropriate antibiotic and incubated at 37^{o}C overnight. Any resultant colonies were taken and colony purified onto fresh LA Km. plates with incubation overnight at 37° C.

P22 transduction was used, as in 2.3.7 to transfer gene mutations into the wild type SL1344 strain of *S.* Typhimurium. External PCR amplification was used to confirm mutagenesis with primers designed externally to the chromosomal loci where the desired mutation should be, as well as primers designed specifically to the antibiotic cassette.

2.8 *In vitro* and *in vivo* assays

In vitro and *in vivo* assays were set up to compare the growth and functioning of mutant *S.* Typhimurium strains with the wild type parent strain.

2.8.1 Growth curves

For analysis of bacterial growth at varying temperatures, *S.* Typhimurium mutant strains and the wild type parent strain were grown overnight at 37°C in 5ml LB broth with the addition of appropriate antibiotics for selection. These cultures were diluted 100 fold the next day into conical flasks with 25 ml of LB broth and cells were incubated at various temperatures under aeration. For ethanol, sucrose and sodium chloride growth curves, overnight cultures were diluted 100 fold the next day into flasks with 25 ml of LB broth with the addition of ethanol, sucrose or sodium chloride to the flasks at varying concentrations and incubated at 37^{o}C under aeration. For all

growth curves, 1 ml samples were removed at one-hour intervals and OD600nm readings were taken. Growth curve experiments were also tested using a Bioscreen C machine (Oy Growth Curves Ab Ltd). For these experiments, overnight cultures were set up as shown above, and diluted 100 fold the next day into 5ml of LB broth. 200µl aliquots were then placed onto a 96 well micro titre plate (Thermo- Hybaid), allowing several replicates to be analysed. Concentrations of H_2O_2, ethanol, sucrose or sodium chloride were added where appropriate. The Bioscreen C machine has integrated software which records OD600nm readings at stipulated time points without disturbing the samples, minimising any contamination errors or growth perturbations caused by the frequent removal of flasks from the incubator for OD 600nm reading.

2.8.2 Disc diffusion assay

Bacteria were grown overnight at 37°C in 5 ml LB broth with the addition of the appropriate antibiotics. The following day a 10-fold dilution was made into 5 ml LB broth and the cultures were grown for 2 hours at 37°C. 100 µl of this was then used to inoculate Top agar (0.7 g agarose in 100 ml LB broth) and poured over pre-warmed LA plates to form a bacterial lawn. Plates were left for approximately half an hour to allow the top agar to set. 6-mm filter paper discs were soaked in 10 µl of either 3% Hydrogen peroxide, or 2% paraquat (methyl viologen) (both Sigma). These discs were then placed on the Top agar surface and incubated overnight at 30, 37 or 42°C. The following day the diameter of the zones of inhibition were measured. 8 replicates were performed for each compound tested. Antibiotic sensitivity was tested as above using various antibiotic discs (Oxoid) covering many different modes of action.

Results were shown as millimetres of inhibition from the perimeter of the disc, or as MIC values, in the case of antibiotic discs.

2.8.3 Inhibition assays

To investigate the effect of bile acids and other compounds on bacterial survival, inhibition assays were set up as follows. Bacteria were grown overnight at 37°C in 5 ml LB broth with the addition of the appropriate antibiotics. Overnight cultures were diluted to a concentration of approximately 2 x 10^{3} CFU/ml, and were subjected to various concentrations of bile acids or CCCP in 96 well micro titre plates. The plates were incubated overnight at 37ºC and the wells of the plates were recorded at OD600nm using a spectrophotometer plate reader.

2.8.4 Invasion of *S.* Typhimurium strains in a macrophage-like cell line

The ability of *S.* Typhimurium mutant strains to invade phagocytic cells was assessed using the macrophage-like cell line RAW264. Cells were routinely cultured in Dulbecco modified Eagle medium (DMEM; Gibco-BRL) supplemented with 4 mM L-glutamine, 10% (vol/vol) foetal bovine serum and 1 X Antibiotic-Antimycotic mix (Gibco-BRL). For invasion assays, cells were seeded into 24-well culture plates (Costar) at 2 x 10^{5} cells per well and incubated overnight at 37°C with 5% CO2. Prior to infection the monolayers were washed twice in antibiotic-free DMEM. Bacteria from overnight culture in LB- glucose (LB-G) broth were diluted in DMEM to give 2

x 10^5 CFU/ml. Then, 1 ml of bacterial suspension was added to each well to give an approximate 1:1 multiplicity of infection. Bacterial invasion was synchronised by centrifugation at 1,000 rpm for 10 min followed by incubation for 1 to 2 hours as described above. The monolayers were then washed twice with sterile PBS (phosphate buffered saline) and overlaid with 1 ml of DMEM containing 100 μg of Gentamicin per ml and incubated for 1 hour as described above. The monolayers were washed twice with PBS, and either the cells were lysed with sterile water or they were overlaid with 1 ml of DMEM containing 10 μg of Gentamicin per ml and incubated for a further 21 hours before being washed and lysed as described above. After lysis of the cells, the number of viable bacteria released from the cells was determined by plating serial dilutions on LA plates.

2.8. 5 Mouse infection models

6 to 8 week old female BALB/c mice (Harlan, UK) were used in all mouse infection studies. To prepare the inoculum for infection, 200mls of bacteria were cultured overnight without shaking. The cells were recovered by centrifugation at 5000rpm for 10 minutes, washed twice in 30mls sterile PBS, and resuspended in 5mls PBS. The concentration of bacteria (CFU/ml) was determined spectrophotometrically and adjusted to the appropriate optical density, prior to animal infection

2.8.5.1 Intraperitoneal challenge

For intraperitoneal (IP) challenge studies, a competition assay method was used (25, 166) to compare the infectivity status of mutant bacteria compared to wild type S. Typhimurium. A minimum group of 3 mice were infected by intraperitoneal injection with 0.2ml of a 10^4 CFU/ml suspension (unless stated otherwise) that consisted of an equal mixture of the two strains being tested. A dilution of the inoculum was plated onto both Km. and Sm. plates for viable counts and accurate determination of the dose of each strain given. Mice were monitored daily, euthanized 72 hours after infection and liver and spleens were recovered. Organs were homogenised in 5ml PBS, using a stomacher (Seward 80). Serial dilutions of each organ were plated onto LB-agar plates containing the appropriate selective antibiotic and viable counts were obtained. The competitive index (CI) was determined from the equation: CI = (output CFU strain A/ output CFU strain B) / (input CFU strain A/ input CFU strain B). A CI of ~1.0 indicates that the strains compete equally well. Statistical significance of the CI was measured using a two tailed unpaired t test.

The competitive index is defined as the output ratio (mutant/wild type) divided by the input ratio (mutant/wild type) and can be determined from the following equation:

CI = (output CFU strain A/ output CFU strain B) / (input CFU strain A/ input CFU strain B).

CI values of around 1 show both strains compete equivalently, with the lower the CI value, the more attenuated the strain under study.

2.8.5.2 Oral Challenge

S. Typhimurium strains that showed significant attenuation following IP infection, were subjected to oral challenge to mimic more accurately the usual route of *Salmonella* infection. To investigate virulence of a strain, inocula containing approximately 5 x 10^7 CFU/ml mutant bacteria or 5 x 10^5 CFU/ml WT bacteria (unless otherwise stated), were prepared and 200µl was administered via oral gavage. It is necessary to give a reduced dose of WT compared to the mutant bacteria as a higher dose of WT would result in immediate death, while if a lower dose of mutant bacteria was administered it may not be possible to observe the effects of the mutation due to the level of attenuation. Mice were culled 7 days later and organs (livers, spleens, Peyer's patches and mesenteric lymph nodes) were isolated and processed as above. Statistical significance was analysed by ANOVA where more than two strains were being compared, or a two tailed unpaired t test for comparison of just two strains.

2.8.5.3 Immunisation studies

Studies were established to investigate the vaccine potential of strains showing significant attenuation in IP and oral studies. To initiate these vaccine studies, for each strain under investigation a group of 8 mice were infected by the oral route of infection with approximately 1x10^9 CFU/ml and following survival these mice were challenged a month later with ~5x10^7 CFU/ml of SL1344 WT *S.* Typhimurium. At this stage a control group of 4 naïve mice which had not undergone pre-immunisation were also challenged with ~5x10^7 CFU/ml of SL1334 WT *S.* Typhimurium. Mice

were monitored and weighed daily and any deaths recorded. Monitoring was performed twice daily, and all mice were graded following a scoring system for mice infected with *Salmonella* species. This system of scoring ensured that mice were monitored more closely if clinical signs of infection appeared, and mice were euthanized when their scoring reached a point where infection was deemed irreversible.

At the end of this experiment, survival was recorded in days for each strain under investigation and this was compared against the control group of mice.

2.9 RNA isolation

RNA was isolated from samples for microarray analysis and Real-time quantitative PCR (RT-PCR) using the Promega SV Total RNA purification kit and following the Institute of Food Research RNA extraction and purification protocol (176). RNAse free plastic ware and solutions were used throughout. 5ml overnight cultures of bacteria were grown at 37ºC with aeration and these cultures were diluted 1/100 into fresh LB broth and grown to an OD600nm of 0.5. Where appropriate, 0.2% arabinose was added to cells for induction of RpoE. Cells were harvested, transferred to a tube containing 1/5 volume 5% ($^{v}/_{v}$) phenol and 95 % ($^{v}/_{v}$) ethanol and allowed to stand on ice for 30 minutes before being centrifuged at 3220xg for 10 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended using the residual liquid. Cells were then transferred to a microcentrifuge tube and centrifuged at 15,000g for one minute. The supernatant was discarded and pellets were frozen at -80ºC overnight. The pellets were then resuspended in 100µL of TE buffer containing 50mg/ml of lysozyme and incubated at room temperature for 5 minutes. All solutions

used throughout the rest of the RNA isolation protocol were supplied with the Promega SV Total RNA purification kit. 75µl of lysis reagent was added to cells and mixed by inversion. 350µl of RNA dilution buffer was then added and mixed well. The samples were heated at 70ºC for 3 minutes and then centrifuged for 10 minutes at top speed. The supernatant from the samples was removed to a clean centrifuge tube and mixed with 200µl of ethanol before being transferred to spin columns. The samples were then centrifuged for 30 seconds at top speed, the elute was discarded and the columns were washed with 600µl of wash buffer. The samples were again centrifuged for 30 seconds at top speed and the elute was discarded. A DNAse mix was then prepared (5µl of 90mM $MnCl_2$, 40µl DNAse core buffer and 5µl DNAse) applied to the column matrix and incubated at room temperature for 15 minutes. Following DNAse treatment, 200µl of DNAse stop mix was added to the column and centrifuged for 30 seconds. The columns were then washed with 600µl of wash buffer, elute was discarded, and columns were given a final wash with 250µl of wash buffer. Columns were transferred to sterile microcentrifuge tubes and 100µl of RNase-free distilled water was added to the column matrix. Samples were allowed to stand for 1 minute before being centrifuged at 4500xg for 2 minutes. RNA was quantified by spectral absorption. 1µl of the RNA sample was diluted 1/100 in TE (10mM Tris HCl pH 8, 1mM EDTA) and the A260 and A260/A280 ratios determined. RNA concentration was calculated using the A260 reading with an A260 of 1 being equivalent to 40µg RNA/ml. Purity was determined by an A260/A280 ratio of between 1.7 and 2.1. From exponential phase cultures approximately 50-60µg of pure RNA was produced.

To further assess the quality of RNA produced where necessary, denaturing agarose electrophoresis was performed with the use of formaldehyde. A 1.2% formaldehyde

gel was prepared in a fume hood with 1.2 g of agarose, 10 ml of 10 x FA gel buffer (200mM 3-[N-morpholino] propanesulfonic acid (MOPS), 50mM sodium acetate, 10mM EDTA to pH 7.0 with Na OH) and RNase free water to 100ml and the mixture was heated until the agarose was melted. 1.44 ml of 37% formaldehyde and Ethidium bromide to a final concentration of 200ng/ml was added, mixed thoroughly and poured into the gel cast. The gel was equilibrated in 1 x FA running buffer (100ml 10 x FA gel buffer, 20 ml 37% formaldehyde, and RNase-free water to 1 litre) for 30 minutes.

To prepare the RNA sample for gel electrophoresis, 5µl of sample loading buffer was made from 10µl 10 x FA gel buffer, 12µl RNase-free water, 50µl de-ionised formamide, 18µl 37% formaldehyde and 10µl of 10 x loading dye (5 ml glycerol, 20 µl 0.5M EDTA, 1ml 0.25% bromophenol blue and 1ml 0.25% xylene cyanol FF to 10 ml with RNase-free water) and mixed with 1µl of RNA. The samples were then incubated for 10 minutes at 65ºC, chilled on ice and loaded onto the equilibrated formaldehyde gel. The gel was run at 70V/cm in 1 x FA gel running buffer.

For microarray studies and RT-PCR, three biological RNA replicates were isolated and samples were stored at -80ºC.

2.10.1 Microarray design and hybridization

RNA labelling and hybridisation was carried out by Maria Fookes (Sanger Institute, Cambridge, uk) onto *S.* Typhimurium LT2 and SL1344 arrays, see chapter 5.

RNA from 4 Cpx associated strains [Gvb822 CpxAΔ, Gvb870 CpxA* (constitutively expressed cpxA) and SL1344 containing plasmid pND18 induced and non-induced to

over-express NlpE] was isolated as in 2.9 and sent to Maria Fookes for further processing.

16µg of RNA was fluorescently labelled with Cy3 or Cy5 dyes (Amersham) during reverse transcription into cDNA using SuperScript^{TM} II reverse transcriptase (Invitrogen). Dye-swap experiments were performed for all hybridizations and in each case SL1344 was used as the reference strain. Labelled cDNA was then purified using an Autoseq^{TM} G-50 column (Amersham), precipitated and the resulting probes hybridized to the *Salmonella* microarray slide overnight at 49ºC. After stringent washing, hybridization results were analysed using a Genepix 4000B scanner (Axon instruments, Inc.) and quantified using Genepix Pro software (Axon Instruments, Inc.). Three biological replicates were performed for each test strain for the two growth phases.

2.10.2 Microarray data analysis

Hybridisation to microarray slides was detected using Genepix 4000B scanner (Axon Instruments, Inc.) and quantified using Genepix Pro software (Axon Instruments, Inc.). Signal intensities were corrected by subtracting the local background. Data were filtered such that spots with a value for median signal intensity minus the background below 50 (in both the reference and test channel) were discarded. Intensity-dependent Lowess normalisation was performed to compensate for unequal dye incorporation. The ln(Cy5/Cy3) ratio intensity of all spots from the hybridisation was calculated and the mean ln(Cy5/Cy3) from up to eight data points per gene (i.e. four slides, duplicate features per slide), was used in all subsequent data analyses. Data were ordered and labelled according to the *S.* Typhimurium LT2 genome generic gene names. The

relationship between strains was calculated by average-linkage hierarchical clustering using the Pearson Correlation as part of the GeneSpring microarray analysis software V5.0 (Silicon Genetics). Exact cut-offs for present/absent genes are often inaccurate for microarray data, therefore the presence/conserved or absence/divergent nature of each gene was calculated by the final calculation ln(Cy5/Cy3) ratio intensity of the normalised data, ultimately giving a standard GeneSpring coloured representation. Those genes that can be considered to be absent/divergent in this experiment with the greatest degree of certainty lie at the blue end of the scale, around an ln(Cy5/Cy3) ratio of 0. Those genes that are present/conserved with the greatest degree of certainty lie in the yellow region and have an ln(Cy5/Cy3) ratio of around 1. The red end of the scale indicates an increased hybridisation with the test strain and may be to due to genes with multiple orthologues; grey represents missing data.

The final readouts were mean Lowess-normalized Cy5/Cy3 ratio intensities for up to 24 data points per gene (i.e. four slides per biological replicate, duplicate features per slide).

2.11 Real-time quantitative PCR

Total RNA was isolated from bacterial strains for RT-PCR as shown in 2.9. For quantitative, real-time, one step RT-PCR, a QuantiTect SYBR Green RT-PCR kit (Qiagen) was used. This kit contains both QuantiTect SYBR green RT-PCR master mix and QuantiTect RT mix, allowing both reverse transcription and PCR to take place in a single tube. All reagents required for both reactions were added at the beginning, eliminating any contamination hazard. The ABI prism 7500 sequence detection system (Applied Biosystems) was used for all RT-PCR reactions. The

SYBR green RT-PCR master mix contains the fluorescent dye ROX, which serves as an internal reference for normalisation of the SYBR Green fluorescent signal when used with the ABI sequence detection systems.

Oligonucleotides were designed for all genes to be investigated which included RpoE and other sigma factors, putative RpoE regulated genes, Cpx two-component system genes, putative cpx regulated genes, internal control genes and several other genes of interest. These are shown in table four. For maximal efficiency, oligonucleotides were designed to be approximately 20bp in length with full homology to the gene of interest, producing an amplicon of approximately 100bp relatively near the start of the gene. Following RNA isolation from the appropriate bacterial strain of interest, samples were set up for RT-PCR to a final volume of 50µl. 25µl of 2 x Quantitect SYBR green RT-PCR master mix, 5µl of forward and reverse oligonucleotides (final concentration 0.5µM), 0.5µl QuantiTect RT mix, 13.5µl RNase-free water and 1µl of RNA (500ng) were added to microamp 96 well optical plates (Applied Biosystems) and placed in the ABI prism 7500. For all RT-PCR reactions studies, three replicates and a negative control were tested. The Real-time cycler conditions were programmed as follows: Reverse transcription cycle at 50ºC for 30 minutes, PCR initial activation cycle at 95ºC for 15 minutes followed by 35 3-step cycles of denaturation at 94ºC for 15 seconds, annealing at 55ºC for 35 seconds and extension at 72ºC for 35 seconds. Primer-dimers may occur depending on primer design and copy number of target; however these can be distinguished from specific products through their lower melting point. A melting curve analysis step was routinely performed to verify the specificity and identity of the RT-PCR products. Dissociation parameters involved one cycle of 95ºC for 15 seconds, 60ºC for 20 seconds and 95ºC for 15 seconds. Where it appeared that primer-dimers were present, oligonucleotides were re-designed

and re-tested. Specificity of RT-PCR products was also checked by agarose gel electrophoresis as shown in 2.4.5. RT-PCR data is acquired during the exponential phase of amplification where reagents are still in excess, and the DNA polymerase is still highly efficient, resulting in more accurate data.

To analyse the RT-PCR data, a quantitative study plate is set up within the ABI prism 7500 software allowing relative quantitation of gene expression For each plate analysed, baseline and threshold values can be altered where appropriate, giving the resulting data in both graphical and numerical format. A comparative cycle threshold (C_T) method uses arithmetic formulas to calculate relative quantitation. Using this method, the amount of target, normalized to an endogenous reference and relative to a calibrator is given by: $2^{-\Delta\Delta C}{}_T$

The equation that describes the exponential amplification of PCR is:

$$X_n = X_0 \times (1 + E_X)^n$$

Where X_n = number of target molecules at cycle n, X_0 = initial number of target molecules, E_x = efficiency of target amplification and n = number of cycles.

The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold:

$$X_T = X_0 \times (1 + E_X)^{C_{T,x}} = K_X$$

Where X_T = threshold number of target molecules, $C_{T,X}$ = threshold cycle for target amplification and K_X = constant.

Relative expression software tool (REST) (293) was used to study relative quantification between groups and also as a test for significance of the results using a statistical model. REST is based on an efficiency corrected mathematical model for data analysis and allows the comparison of two groups, with up to 16 data points in

the sample group versus 16 points in the control group, and tests the group differences for significance using a randomisation test.

Table four - A list of oligonucleotides designed and used throughout this study.

Oligonucleotide	Sequence 5' to 3'	Function
pspAFF	cccaagttaattcaatcctcacataat	lacZ promoter fusion
pspAFR	gctctagagcgatgaaaatcgcacttgt	lacZ promoter fusion
pspFAF2	gctctagcgcaattcaatcctcacataat	lacZ promoter fusion
pspFAR2	cccaagcttggggatgaaaatcgcacttgt	lacZ promoter fusion
pspFARlong	gctctagagcaatcagcactggcttatcca	lacZ promoter fusion
pspFAFlong	Cccgtcggcaaaacgagaaaagcttgggcgat	lacZ promoter fusion
pspCextFor2	Acgccggcaggcccgtagcg	external PCR check of pspC mutant
pspCextR	Gaagacattcttgatgcaga	external PCR check of pspC mutant
pspextF2	caactacccggagagatcaacaacg	external PCR check of psp mutant
pspextR2	ctacttagtagacccgctatcacga	external PCR check of full psp mutant
htrAFWCL	Cgagattgaaacacatggcgaaaaccacattagc	amplifying htrA gene for htrA gene/ promoter constructs
htrAFWproCL	agccgctattaagcttgtcgcttaacgac	amplying htrA promoter for gene/ promoter constructs
htrAREVproCL	Tgtggttttcgccatgtgtttcaatctcgattaac	ampifying htrA promoter for gene / promoter constructs
htrArv1bamhi	Cgggatccttactgcattaacaggtagatggtg	Amplification of variant sections of htrA gene
ddgREDFor2	Gaaggtatttttatgtttcctcaaagcaaatttcacgcgcgtgtgtaggctggagctgcttc	ddg RED mutagenesis
ddgREDrev	cctttttcagatgtagagtgacgcttccccc aacgggcgacatatgaatatcctccttag	ddg RED mutatgenesis
slyBREDFor	Gggtaaatgattaaacgtgtactggccgttcactgatgggtgtqaggctggagctgcttc	slyB RED mutagenesis
slyBREDRev	cctggtttagcgtggagagacggtaacctggctgccgttgcatatgaatatcctccttag	slyB RED mutagenesis
NlpBREDRev	gttaaattacttgttgaacgctgcctggaagacggcaacccatatgaatatcctccttag	nlpB RED mutagenesis
NlpBREDFor	Gatttgatggcttactcagtacaaaagtcgcgcctggcgagtgtaggctggagctgcttc	nlpB RED mutagenesis
smpAREDFor	Atcactatgcgctgtaaaacgctgactgctgccgcagcaggtgtaggctggagctgcttc	smpA RED mutagenesis

Oligonucleotide	Sequence 5' to 3'	Function
smpAREDrev	acaaaattacttcgtcaacgccggtttgttatcaatattgcatatgaatatcctccttag	smpA RED mutagenesis
ibpAREDfor	atgattatgcgtaactttgatttatccccgctgtaccgttgtgtaggctggagctgcttc	ibpA RED mutagenesis
ibpAREDRev	acggaatcagttaatttcgatacggcgcggtttgttcgctcatatgaatatcctcctag	ibpA RED mutagenesis
ibpBREDfor	atgattatgcgtaactacgatttatccccactgctgcgtcgtgtaggctggagctgcttc	ibpB RED mutagenesis
ibpBREDRev	agctcattagctatttaatgcggagcgttcgttaatggcgcatatgaatatcctccttag	ibpB RED mutagenesis
ychHREDFor	tgacatatgaaacgcaaaaacgcttcgttattcggtaacggtgtaggctggagctgcttc	ychH RED mutagenesis
ychHREDRev	aaccggttagctatgacgacgattgtcgttacggcggcaacatatgaatatcctccttag	ychH RED mutagenesis
yfgMREDFor	Gacagcgtggaaatttacgagaacgaacacgaccaggtgttgtgtaggctggagctgcttc	yfgM RED mutagenesis
yfgMREDRev	tccctctcagatggacaaattattgattttcatctgcatccatatgaatatcctccttag	yfgM RED mutagenesis
PqiAREDFor	Gcgcctatgtgtgaacatcaccatgctgcgaagcacatatgtgtaggctggagctgcttc	pqiA RED mutagenesis
PqiAREDRev	atgctatcaggactcctcatggcctggttcgtactcacgacatatgaatatcctccttag	pqiA RED mutagenesis
pspAredF	tgaattatgggtatttttctcgttttgccgacatccgtagtgtaggctggagctgcttc	pspA RED mutagenesis
pspAredR	tgtcattattgattatcttgcttcattttggctttcaactcatatgaatatcctccttag	pspA RED mutagenesis
ibpABredF	Tcttagaaggagaaatgattatgcgtaactacgatttaatcgtgtaggctggagctgcttc	ibpAB RED mutagenesis
ibpABredR	aaggcaaggagctcattagctatttaatgcggagcgttcgcatatgaatatcctccttag	ibpAB RED mutagenesis
rpoNredF	Tagcgggagaaaacgactctgaatatgaagcaaggtttgcgtgtaggctggagctgcttc	rpoN RED mutagenesis
rpoNredR	gttgggtcaaaccagctgtttgcgttggtttgacggcggacatatgaatatcctccttag	rpoN RED mutagenesis
htrApromGenF	aagcttggtaccgcttaacgactttcgcg	for general promoter vector (htrA promoter in pWSK29)
htrApromGenR	atcgatccatggtgtttcaatctcgatta	for general promoter vector (htrA promoter in pWSK29)
ddgextF	Agtaagtgcttcggataagc	external PCR check of ddg mutant

Oligonucleotide	Sequence 5' to 3'	Function
ddgextR	Taaaaaatcgcctgtaaaca	external PCR check of ddg mutant
slyBextF	Acattctttcggcagaagat	external PCR check of slyB mutant
slyBextR	Cttctgattaaacttatcgc	external PCR check of slyB mutant
nlpBextR	Ctgtaattgtggtgcgaaat	external PCR check of nlpB mutant
nlpBextFor2	Cagcgcttcagcatgctggc	external PCR check of nlpB mutant
smpAextF	Cgccgtaagacccgacagca	external PCR check of smpA mutant
smpAextR	Taagccagataaacgtagcg	external PCR check of smpA mutant
ibpAextF	Tataagacgtagtgaataga	external PCR check of ibpA mutant
ibpAextR	Ccttctaagaagcgagtttt	external PCR check of ibpA mutant
ibpBForEXT	Tcgggtcgcgttgcgcggcc	external PCR check of ibpB mutant
ibpBRevEXT	Ctcatgttagaaaaccgccc	external PCR check of ibpB mutant
ychHForEXT	Gatcgcacccgcacttacaa	external PCR check of ychH mutant
ychHFRevEXT	Tgggcgtctgaccttcttcc	external PCR check of ychH mutant
yfgMForEXT	Actgttgtagtgaaggattt	external PCR check of yfgM mutant
yfgMRevEXT	Cagtgaacagccgctgagca	external PCR check of yfgM mutant
pqiAForEXT	Gcaagccttcgaacgttggg	external PCR check of pqiA mutant
pqiAextRev2	Ttgtactttggcttcccctt	external PCR check of pqiA mutant
rpoNextF	Gctatgctgctctagcggga	external PCR check of rpoN mutant
rpoNextR	Ctgattaatccgctcaaaat	external PCR check of rpoN mutant
pspAEXTFor	Gtggtatgaaaagttggcac	external PCR check of pspA mutant
pspAextRev	Acctaaagctgatgagcgac	external PCR check of pspA mutant
pspREDF	gattatctatttttttactttcggcatatcaag acgactggtgtaggctggagctgcttc	psp mutagenesis
pspREDR	aaatagctaaagctgatgctttttaagcaa ggcgcgaaaccatatgaatatcctccttag	psp mutagenesis
degSRTfor	Tcgccgtcccgcaattcgac	degS RT PCR

Oligonucleotide	Sequence 5' to 3'	Function
degSRTfor	Tatgcgcggtactgttcata	degS RT PCR
cpxARTfor	Tggacagcgaacagcgccag	cpxA RTPCR
cpxARTrev	Taaccgctgtccaggcggcg	cpxA RTPCR
cpxRRTfor	gcccacgacggcgagcaggc	cpxR RTPCR
cpxRRTrev	Agcataatgacaggcgtctg	cpxR RTPCR
cpxPRTfor	Cattcgttgctgatgctttt	cpxP RTPCR
cpxPRTrev	Aaaatggcgcaagagcaggt	cpxP RTPCR
rpoERTfor	Tagtgcgctaccagcataaa	rpoE RTPCR
rpoERTrev	Cccggaaagaatccagcgcg	rpoE RTPCR
rpoHRTfor	Gttatcggctgacgaggagc	rpoH RTPCR
rpoHRTrev	Tagttacgagcaatatgaac	rpoH RTPCR
htrARTfor	Gcggctgaaacgtcctcttc	htrA RTPCR
htrARTrev	Attcaccgtggtgctacctt	htrA RTPCR
fliCRTfor	Ttaaagcctcggctactggt	fliC RTPCR
fliCRTrev	Ggaaacttcataatagccat	fliC RTPCR
fljBRTfor	Gatgatgcagctattaaagc	fljB RTPCR
fljBRTrev	Tttttggcggcatcagcacc	fljB RTPCR
relARTfor	Tggatcgcaagcctgggaat	relA RTPCR
relARTrev	Agaatttctaccatctccac	relA RTPCR
rplARTfor	Gacatcaacgaagctattgc	rpl RTPCR, internal control
rplARTrev	Ttctggtcatgatttacgagc	rpl RTPCR, internal control
pspFRTfor	Tttcttgaagtactggaaca	pspF RTPCR
pspFRTrev	Tgaagagagataatgcagtc	pspF RTPCR.

Oligonucleotide	Sequence 5' to 3'	Function
pspARTfor	Caatatcaatgcgttgttgg	pspA RTPCR
pspARTrev	Tccgctaaagctcgcgcgga	pspA RTPCR
smpARTfor2	Gatgttgaccgcaggctgtt	smpA RTPCR
smpARTrev2	Tgcctaaagcatacgcaacc	smpA RTPCR
pspBRTFor2	Gttaccgatttggctgtggc	pspB RTPCR
pspBRTRev2	Cagcgcctgaatgcgctcgc	pspB RTPCR
lrhART2for	Cgatctgctgagaacgttcg	lrhA RTPCR, internal control
lrhARTrev2	Acgagttgctcaagacgctg	lrhA RTPCR, internal control
nmpCRTfor	Cagccgaggtatataacaaa	nmpC RTPCR, internal control
nmpCRTrev	Tcgcctttaaagcccagacg	nmpC RTPCR, internal control
rpmCRTfor	Gaacaccgagctactgaacc	rpmC RTPC, internal control
rpmCRTrev	Cgacatcacgacgcacttgc	rpmC RTPCR, internal control
lppRTfor	Ctgtcttctgacgttcagac	lpp RTPCR, internal control
lppRTrev	Ttagcgcgagctgcgtcgtc	lpp RTPCR, internal control
csgARTfor	Ctctggctggcgtcgttcca	csgA RT PCR. Fimbrial gene
csgARTrev	Gttagcggaaccgtactgat	csgA RT PCR. Fimbrial gene
fimARTfor	Agcgtgagtggcggtactat	fimA RTPCR. Fimbrial gene
fimARTrev	Cgtaaagctggcggtacggt	fimA RTPCR. Fimbrial gene
stdARTfor	Tttcattaataccctcagag	stdA RTPCR. Fimbrial gene
stdArRTrev	Tacatcataccggcagccgc	stdA RTPCR. Fimbrial gene
sthARTfor	Caataatgatgagtcgtcaa	sthA RTPCR. Fimbrial gene
sthARTrev	Taaacgcctgcttatttgtg	sthA RTPCR. Fimbrial gene
stbARTfor	Aagtctctgatgaaacatgc	stbA RTPCR. Fimbrial gene
stbARTrev	Atatcaaacgttattggccc	stbA RTPCR. Fimbrial gene

Oligonucleotide	Sequence 5' to 3'	Function
bcfARTfor	Cggatactacaaccgtcact	bcfA RTPCR. Fimbrial gene
bcfARTrev	Gttaatttactggcgcgaac	bcfA RTPCR. Fimbrial gene
stcARTfor	Ctgttgatgagtatgattca	stcA RTPCR. Fimbrial gene
stcARTrev	Accaatttcattcagtctaa	stcA RTPCR. Fimbrial gene
lpfARTfor	Agtttccacttctgctttcg	lpfA RTPCR. Fimbrial gene
lpfARTrev	Cttaacctgacccagcacaa	lpfA RTPCR. Fimbrial gene
ychHTRfor	Ttattcggtaacgtgctgat	ychH RTPCR.
ychHRTrev	Atgctcagtattgcgccatg	ychH RT PCR
PSPCREDF	Tatcacttacagttgacggaaacgactac gcagtgtgaacgtgtaggctggagctgct tc	pspC RED mutagenesis
PSPCREDR	gcgctaattgggtggaatcaatctgaataa aaaactatggccatatgaatatcctccttag	pspC RED mutagenesis
stm3030RTFor	Actgataacgttactctgaa	stm 3030 RTPCR
stm3030RTRev	Cagttgttccgccttttggg	stm 3030 RTPCR
stm3377RTFor	Ggctttttttatgcagcgcc	stm3377 RTPCR
stm3377RTRev	Ggacgatcgttgcctttaaa	stm3377 RTPCR
stm3378For	Ctgcatcttgccggtttttt	stm3378 RTPCR
stm3378Rev	Aaatttgagcgcggttctgc	stm3378 RTPCR
stm4519RTFor	Tcaacacggctgtcgatcaa	stm4519 RTPCR
stm4519RTRev	Cggacgcggatattgaagcc	stm4519 RTPCR
tolQRAredFOR	tgtcgcggagtttaagcagtgactgacatg aatatccttggtgtaggctggagctgcttc	tolQRA RED mutagenesis
tolQRAredREV	tatctacagtttaaagtctagtttggcatcct ttattttccatatgaatatcctccttag	tolQRA RED mutagenesis
tolBpalybgFredFOR	Gcccaggtatgggagatattgatgaagc aggcattacgagtgtgtaggctggagctg cttc	tolBpalybgF RED mutagenesis
tolBpalybgFredREV	cgcattacatcgcgttaagacgcttctgcg cctgtttcgccatatgaatatcctccttag	tolBpalybgF RED mutagenesis

Oligonucleotide	Sequence 5' to 3'	Function
fliYredFOR	Aacattattgggtcacgtcagcgccaaacattttcagagaggtgtaggctggagctgcttc	fliY - fliR RED mutagenesis
fliRredREV	aaattatgggttattatttatcggcatctcgctaacaatacatatgaatatcctccttag	fliY - fliR RED mutagenesis
fliCREDfor	Ttgataaggaaaagatcatggcacaagtcattaatacaaagtgtaggacggagctgcttc	fliC RED mutagenesis
fliCREDrev	Ggattaacgcagtaaagagaggacgttttgcggaacctggcatatgaatatcctcctag	fliC RED mutagenesis
fljBREDfor	aggaaaattttatggcacaagtaatcaacactaacagtctgtgtaggctggagctgcttc	fljB RED mutagenesis
fljBREDrev	Aaattaacgtaacagagacagcacgttctgcgggacctggcatatgaatatcctcctag	fljB RED mutagenesis
tolBREDFor	attattatcacagatacggcgaccaggcaggcgatttcaccatatgaatatcctccttag	tolB RED mutagenesis
fliCextFOR	Aaccgtgggcaacagcccaa	external PCR check of fliC mutant
fliCextRev2	Agctttcgctgccttgattg	external PCR check of fliC mutant
fljBforext3	Aggaaatatcatttacagcc	external PCR check of flijB mutant
fljBrevext3	Tagtagccatgattttctcc	external PCR check of fljB mutant
TolBextFor	Aacattctgctaaattatcg	external PCR check of TolB mutant and tolBpalybgF mutant
ybgFextRev2	Gcgaccagaaaaaaggcgtt	external PCR check of tolB mutant and tolBpalybgF mutant
CLkanFor	Tgggtggagaggctattcgg	external PCR check for kanamycin cassette
CLkanRev	Gggtcacgacgagatcctcg	external PCR check for kanamycin cassette
ppiAREDfor	Aatctcatgctcaaatcgactctggcggctgtcgcagctggtgtaggctggagctgcttc	ppiA RED mutagenesis
CLkanRev	Gggtcacgacgagatcctcg	external PCR check for kanamycin cassette
ppiAREDfor	Aatctcatgctcaaatcgactctggcggctgtcgcagctggtgtaggctggagctgcttc	ppiA RED mutagenesis

Oligonucleotide	Sequence 5' to 3'	Function
ppiAREDrev	catgttttatggcaggactttcgcggaaag gataactggtcatatgaatatcctccttag	
ppiDREDfor	Tacaccatgatggacagcttacgcacgg ctgcaaacagtcgtgtaggctggagctgc ttc	ppiD RED mutagenesis
ppiDREDrev	Cgacgattactgctgttccagagcgtcgc cgattttgattcatatgaatatcctccttag	ppiD RED mutagenesis
TolQRAextFOR	Actcaaaatgaagcctcgtg	external PCR check of TolQRA mutant
TolQRAextREV	Tgttcagttgcatttcttta	external PCR check of TolQRA mutant
chlorFORcheck	Cacatcttgcgaatatatgt	external PCR check for chloramphenicol cassette (pKD3)
ppiAextFor	Caggaaggctctttaaaatc	external PCR check of ppiA mutant
ppiAextRev	Caggttacgctgagacaggg	external PCR check of ppiA mutant
ppiDextFor	Actcggtagccagcggagaa	external PCR check of ppiD mutant
ppiDextRev	aaaaggccgcgaaagcggcc	external PCR check of ppiD mutant
tolQRTfor	Agcatcctgggccatcatta	tolQ RTPCR
tolQRTrev	Ctttcctggtacagacgcga	tolQ RTPCR
tolBRTfor	Cctgaagatatcggtggcat	tolB RTPCR
tolBRTrev	Tgcggtaggctgaacttcct	tolB RTPCR
pspGREDFor	gcgactatgctggaactacttttgtgcttg gctttttcgtgtaggctggagctgcttc	pspG RED mutagenesis
pspGREDRev	aataccttagtaaaaccgacggttattgcg ctgatactgtcatatgaatatcctccttag	pspG RED mutagenesis
pspGextFor2	Ttgtaagggcttacctgacc	external PCR check of pspG mutant
pspGextRev2	Ttcctatttgcttgtgaggg	external PCR check of pspG mutant
skpRTfor	Gtaacgtccgcacaggctgc	Skp RTPCR
skpRTrev	Attggatacacccgtcttct	Skp RTPCR

Chapter Three - Identification and characterisation of RpoE regulated genes in *S.* Typhimurium

3.1 Introduction

Bacteria have numerous stress response systems in place to survive the many stresses encountered both within the host and the outside environment. The gram negative bacterial envelope comprises an inner cytoplasmic membrane and outer membrane separated by the soluble periplasmic space which helps maintain the bacterial shape. The envelope stress response systems are of particular importance for the efficient maintenance of physiological processes such as protein folding, cell wall biosynthesis, motility, attachment, cell division and pathogenesis in gram negative bacteria.

To date in *E. coli*, there have been three identified extracytoplasmic stress responses (ESR): RpoE (an extracytoplasmic function sigma factor), CpxRA and BaeSR (both two component regulatory systems) and homologues of these systems have also been shown to be present in *S.* Typhimurium (254, 311, 316, 332). The phage shock response is now thought to be a fourth ESR, and this will be discussed in more detail in chapter 4. Downstream of the *rpoE* gene, are *rseA, rseB* and *rseC* which together comprise the *rpoE* operon and allow positive and negative regulation of this ESR. *rseA* is a membrane bound anti-sigma factor that binds to RpoE under non-stressed conditions to dampen RpoE activity. In response to outer membrane stress, *rseA* is cleaved from RpoE and degraded within the cytoplasm, allowing RpoE to complex with the core RNA polymerase and activate transcription of RpoE dependent genes. Many RpoE regulated genes are known to induce vital homeostatic responses such as efficient protein folding preservation by periplasmic proteases and folding factors, and phospholipid and lipopolysaccharide protein biosynthesis. Although many RpoE regulated genes are thought to function to override envelope protein folding

disruption, many remain whose function is unknown. These include a number of small membrane proteins which have not been previously characterised. Interestingly, mutagenesis studies within the Roberts lab have shown the RpoE system to be non-essential in *S.* Typhimurium (under normal laboratory conditions) in contrast to *E. coli* (167) and further studies have been done within our lab to characterise the *S.* Typhimurium RpoE regulon (331).

Extensive characterisation of the *Salmonella* RpoE regulon was performed in the Roberts lab using several approaches. In collaboration with the Kormanec lab, a well-established two-plasmid screening technique (322) was devised to identify putatively RpoE regulated genes (349) and confirmation was established using S1 nuclease mapping (256) to identify relevant RpoE dependent promoters.

To further characterise the RpoE regulon, microarray technology was utilised in collaboration with Dr Jay Hinton's group at the Institute of Food Research (IFR). Total RNA was isolated from wild type SL1344 over-expressing RpoE under the control of an arabinose inducible promoter (SL1344 pAC-RpoEST4) and compared with the same strain containing empty vector. RNA was then labelled and hybridised on *S.* Typhimurium LT2a arrays.

Using these approaches, a comprehensive list of putatively RpoE regulated genes has been established. Table five shows some of the important genes identified from these and other approaches and includes the nine genes initially selected by myself for further study.

Table five - A list of some putative *S.* Typhimurium σ^E regulated genes identified by the two plasmid screen or from the IFR microarray (reference as before) including those selected for further study.

Common gene name	**Description**	**2- plasmid screen**	**IFR microarray ratio**
rpoE	σE ECF sigma factor	yes	111.6
rpoH	σ^{32} heat shock sigma factor	yes	7.2
rpoD	σ^{70} sigma factor	yes	5.16
htrA	Periplasmic serine protease	yes	32.8
surA	Peptidyl – prolyl – cis - trans isomerase	yes	5.92
tolR	Inner membrane protein	yes	4.3
slyB	Putative outer membrane lipoprotein	no	1.23
nlpB	Lipoprotein 34	no	7.91
smpA	Small membrane protein A	yes	7.70
ibpA	Small heat shock protein	no	6.57
ibpB	Small heat shock protein	no	7.04
yfgM	Putative inner membrane protein	yes	4.64
ychH	Putative inner membrane protein	no	4.66
pqiA	Paraquat inducible protein	no	1.94
ddg	Palmitoleoyl transferase	yes	5.80

3.2 Promoter consensus search

New members of the *E. coli* σ^E regulon were identified by a two-plasmid system (323), which allowed for the identification of promoters recognised by a specific sigma factor of RNA polymerase. This method identified 11 new RpoE dependent promoters in *E. coli* (*fusA, ddg, psd, sixA, bacA, sbmA, smpA, yeaY, ybaB, yiiS, and yfeY*).

As with *E. coli*, *S.* Typhimurium RpoE expression is controlled by three promoters, one of which, rpoEp3, is recognised by RNA polymerase holoenzyme containing σ^E. The two-plasmid system utilised to identify new RpoE regulated genes in *E. coli* was optimized for the identification of promoters recognised by *S.* Typhimurium σ^E (349). This data resulted in the identification of 34 σ^E –dependent promoters which controlled the expression of 62 genes including *ddg* and *smpA*.

To look for further RpoE regulated promoters, a *S.* Typhimurium LT2 genome wide promoter consensus search based on the confirmed σ^E regulated *rpoE* promoter (*rpoEP3*) and the first few putative σ^E dependent promoters identified by the two plasmid screen was carried out by myself. A Colibase (51) mismatch pattern search was performed using a specific nucleotide sequence based on previous RpoE promoter knowledge. The coliBASE program was used to search *S.* Typhimurium LT2 for the pattern:

BVAACYW 16N TCNNA

A 16bp nucleotide spacer between the -35 and -10 binding regions was kept constant, as reduction of this spacer to 15bp has been shown to reduce the activity of the *rpoEP3* promoter (255).

RpoE dependent promoters were identified for genes *rpoE, htrA* and *rpoH* as expected and putative promoters were also identified for several other putative RpoE regulated genes characterised further in this chapter. Table six shows a list of genes identified either by the two-plasmid system in collaboration with Jan Kormanec, or through the Colibase mismatch pattern search.

Table six - A list of selected genes for which a putative RpoE regulated promoter could be identified. The location of the promoter is given with respect to the predicted start codon of the given gene.

Gene name	Promoter sequence -35 N(16) -10	Promoter location
rpoE	GGAACTT TCTAA	-110
rpoH	TGAACTT TCTGA	-120
htrA	GGAACTT TCTGA	-73
nlpB	GGAACTT ATCAA	-402
smpA	TAAACTT TCTGA	-151
ychH	CGCACTT TCTAT	-87
ddg	GGAACCA CCTAA	-186
yfgM	GGAATTT TCGAT	-326
pqiA	TGAACCA TCGAA	-528

3.3 Quantitative Real Time RT-PCR of RpoE regulated genes

To further investigate the regulation of two RpoE regulated genes under characterisation in this chapter, quantitative RT-PCR was performed as shown in 2.11. It was chosen as a sensitive method allowing the detection and quantification of gene expression levels of various different genes in several RNA samples. RNA was made from SL1344 WT, SL1344 *rpoEΔ* and SL1344 WT containing the arabinose induced over expressing RpoE plasmid (pAC7-rpoEST4) as shown in 2.9. Oligonucleotides were designed for the genes *rpoE, skp* (a known RpoE regulated gene, (66)) *smpA* and *ychH* using oligonucleotides rpoERTFor, rpoERTRev, skpRTfor, skpRTrev, smpARTfor2, smpARTrev2, ychHRTfor, ychHRTrev as shown in table four. Following a one-step quantitative RT-PCR protocol, relative quantification analysis was performed. Figure 3.1 shows an example of the data obtained. Raw RT-PCR data was then used as in-put data in the relative expression software tool (REST) (293) to study relative quantification between groups and also as a test for significance of the results using a statistical model. RT-PCR studies showed an increase in the expression of all 4 genes tested in the WT SL1344 pAC7-rpoEST4 strain compared to WT SL1344. Figures 3.2 and 3.3 show the REST data for *smpA*, *ychH*, *skp* and *rpoE* itself. This increase in expression corroborates well with our theory that these genes are RpoE regulated, and exhibit increased expression in response to raised cellular levels of RpoE. As such, these studies support the data obtained from the microarray showing gene regulation in an over expressing RpoE strain compared to wild type.

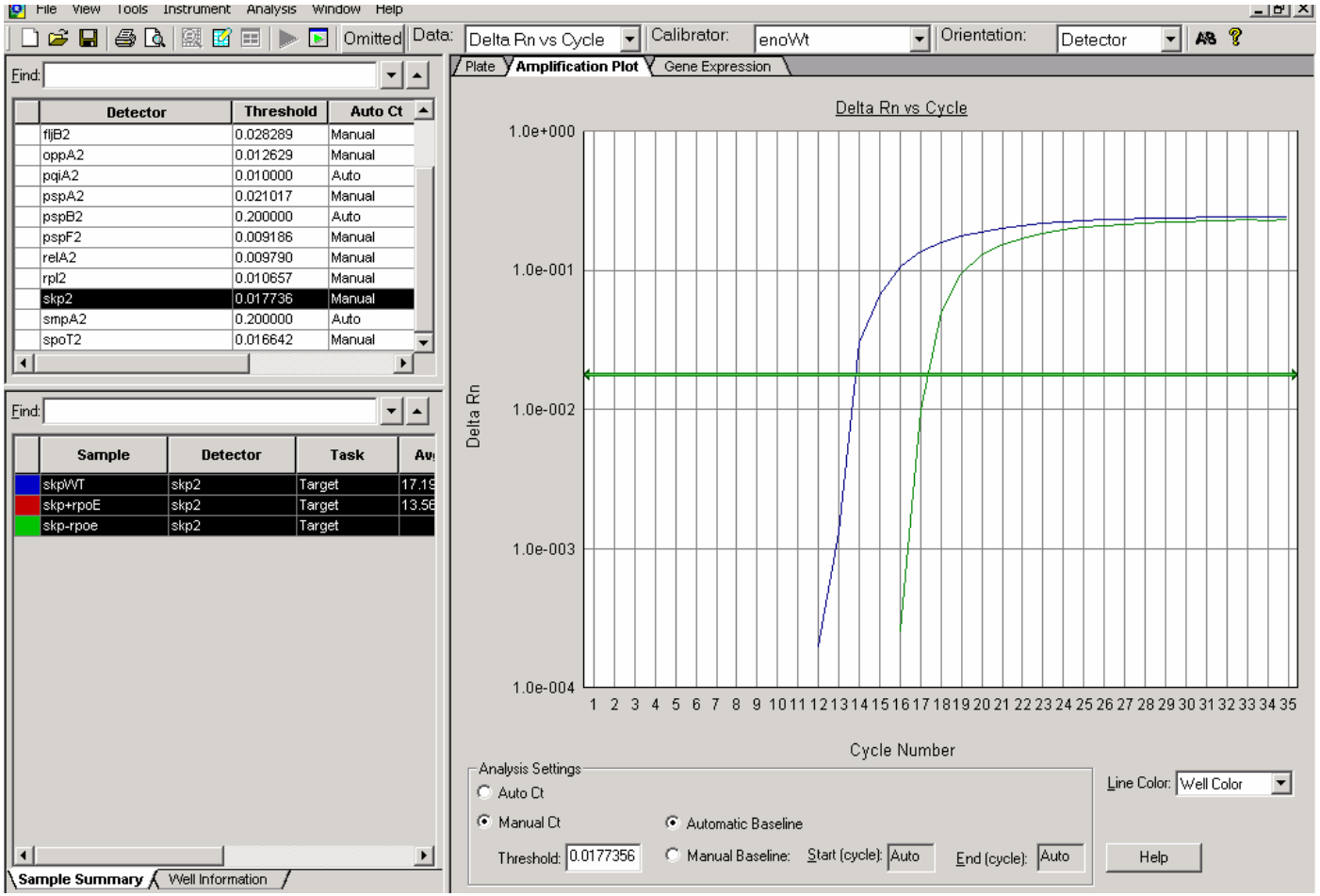
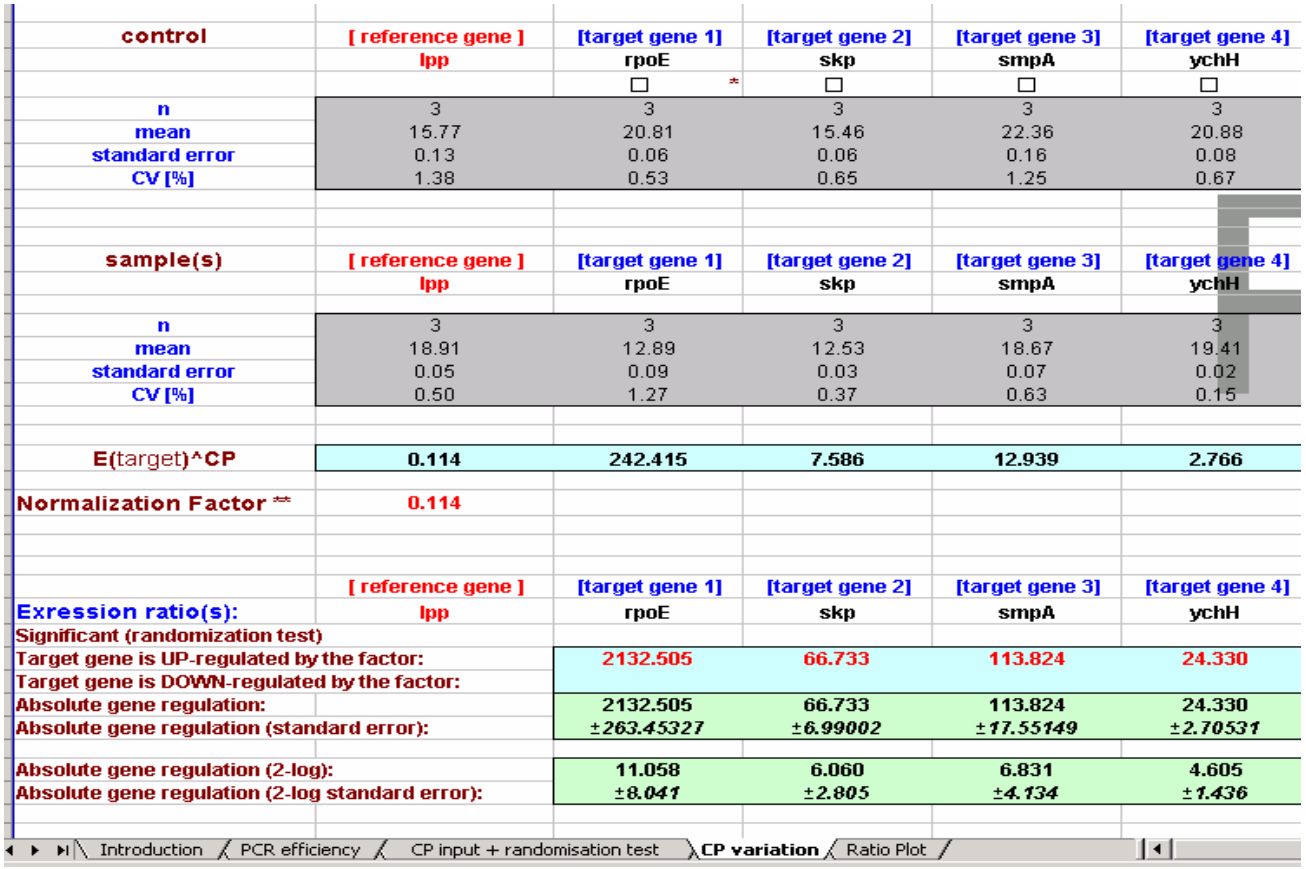


Figure 3.1 - Ilustration of RT-PCR analysis using ABI system software.
Illustration shows the RT-PCR profile obtained when looking at the relative expression of skp in three RNA types (WT, overexpressing RpoE and *rpoEΔ S.* Typhimurium).



control	[reference gene]	[target gene 1]	[target gene 2]	[target gene 3]	[target gene 4]
	lpp	rpoE	skp	smpA	ychH
		☐ *	☐	☐	☐
n	3	3	3	3	3
mean	15.77	20.81	15.46	22.36	20.88
standard error	0.13	0.06	0.06	0.16	0.08
CV [%]	1.38	0.53	0.65	1.25	0.67

sample(s)	[reference gene]	[target gene 1]	[target gene 2]	[target gene 3]	[target gene 4]
	lpp	rpoE	skp	smpA	ychH
n	3	3	3	3	3
mean	18.91	12.89	12.53	18.67	19.41
standard error	0.05	0.09	0.03	0.07	0.02
CV [%]	0.50	1.27	0.37	0.63	0.15
E(target)^CP	0.114	242.415	7.586	12.939	2.766
Normalization Factor **	0.114				

Exression ratio(s):	[reference gene] lpp	[target gene 1] rpoE	[target gene 2] skp	[target gene 3] smpA	[target gene 4] ychH
Significant (randomization test)					
Target gene is UP-regulated by the factor:		2132.505	66.733	113.824	24.330
Target gene is DOWN-regulated by the factor:					
Absolute gene regulation:		2132.505	66.733	113.824	24.330
Absolute gene regulation (standard error):		*±263.45327*	*±6.99002*	*±17.55149*	*±2.70531*
Absolute gene regulation (2-log):		11.058	6.060	6.831	4.605
Absolute gene regulation (2-log standard error):		*±8.041*	*±2.805*	*±4.134*	*±1.436*

Introduction / PCR efficiency / CP input + randomisation test / CP variation / Ratio Plot /

Figure 3.2 - REST (relative expression software tool) displaying RT-PCR data
Expression levels of genes *rpoE, skp, smpA* and *ychH* in WT RNA compared to RNA from an overexpressing RpoE strain. lpp gene is an internal control

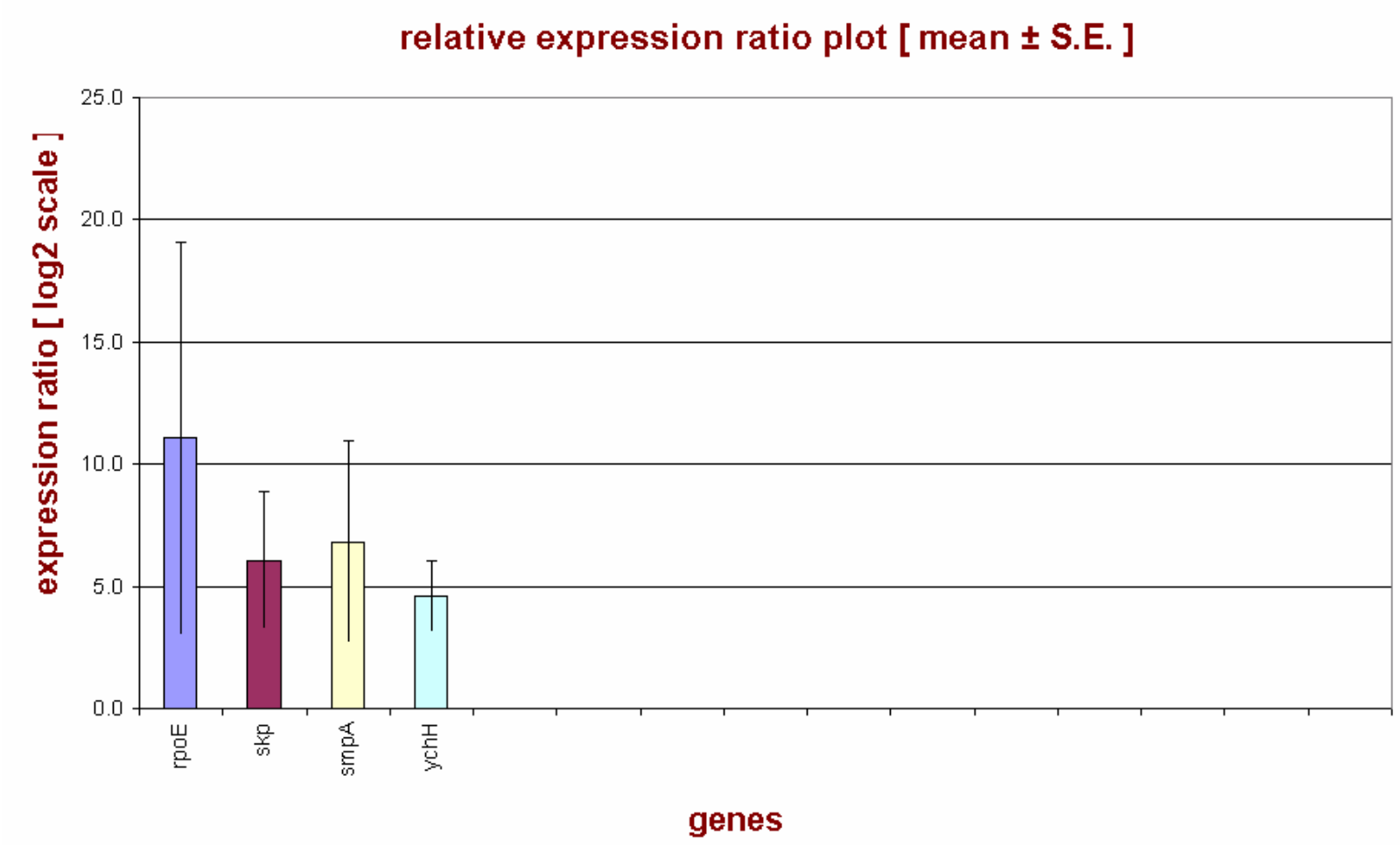


Figure 3.3 - REST (relative expression software tool) displaying relative expression ratio plot. Expression levels of genes *rpoE, skp, smpA* and *ychH* in WT RNA compared to RNA from an overexpressing RpoE strain. *lpp* gene is an internal control

3.4 Mutagenesis of RpoE regulated genes

The RpoE regulon of *S.* Typhimurium was characterised by microarray and promoter searching methods, following which a number of genes were selected by myself from those identified. These genes are *slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA, ddg* and the phage shock genes. The phage shock response has recently been discovered as an important regulatory system in *S.* Typhimurium and will be discussed in more detail in chapter four. *nlpB, smpA, yfgM, ychH, pqiA,* and *ddg* are all proteins for which a RpoE dependent promoter could be found (table six) and showed a high level of up-regulation in the over expressing RpoE microarray, indicating they may play a significant role in the RpoE stress response. *ibpA* and *ibpB*, were also selected for further study as these two genes are highly up-regulated in our array and are thought to be homologues of the genes *stm1250* and *stm1251* which are presumed to play an important role in the RpoE regulon of S. Typhimurium (Gary Rowley, data not shown). The final gene selected for study was *slyB*.

Current knowledge on each of these genes is discussed below and the aim of this chapter was to try and elucidate more fully the role of these proteins whose functions remain largely unknown.

IbpAB

Small heat shock proteins are thought to help the cell adapt to heat shock under adverse conditions. IbpAB are two such small heat shock proteins that appear to be RpoE regulated from data shown above and as such were chosen for further study. IbpA and B share significant similarity with *STM1251* (32 and 31% identity respectively) a *Salmonella* specific gene thought to encode a small heat-shock protein.

STM1250 and *1251* were both identified as RpoE regulated through the identification of two *S.* Typhimurium RpoE dependent promoters (349).

In *E. coli* IbpAB were shown to reduce the accumulation of stress proteins and prevent the degradation of disaggregated proteins at the expense of impaired inclusion body removal (210) however little else is known about the specific role of these proteins in *S.* Typhimurium or other bacteria.

NlpB

NlpB is a lipoprotein which is likely to be involved in outer membrane protein biogenesis. It appeared to be highly up-regulated in our over-expressing RpoE microarray, although its specific role is unknown (281). NlpE is a lipoprotein known to be a strong inducer of another ESR system (the CpxAR two component system) (331) which lends weight to our quest to further investigate the RpoE regulatory potential of *nlpB*. It is also known to be a member of the YaeT complex which is required for outer membrane protein assembly (399).

Ddg

S. Typhimurium Ddg is a 300bp lipoprotein also known as LpxP that shares approximately 90% homology with *E. coli* LpxP. It is a cold-shock induced palmitoleoyl transferase, and is thought to be a putative small heat shock protein in *E. coli, Yersinia* and *Shigella* species (45). It is known to be involved in LPS modification which is important to the pathogenesis of bacteria. Lipoproteins are important in maintaining membrane integrity and there are 6 lipoproteins known to be σ^E regulated in *E. coli* (281). Studies have also shown lipoproteins A and B (LppA and LppB) of *S.* Typhimurium to be highly attenuated when tested with *in vitro* and *in*

vivo models of *Salmonella* pathogenesis (97). It is hoped that further study will elaborate upon the role of *ddg* in the RpoE ESR of *S.* Typhimurium.

SlyB

SlyB is a putative outer membrane lipoprotein conserved in gram negative bacteria including *S.* Typhimurium. It is a homologue of Pal lipoproteins, and thought to play a role in the maintenance of the outer membrane by responding to the presence of magnesium. SlyB is also thought to be regulated by PhoPQ which is a two-component regulatory system that responds to environmental magnesium deficiency (208). Studies have shown *slyB* mutants in *Burkholderia multivorans* to have altered cell morphology and an increased sensitivity to SDS and EDTA (297). Study of the potential role of *slyB* in the RpoE regulon of *S.* Typhimurium would more fully elucidate its overall role in maintaining membrane integrity under varying environmental conditions.

YfgM, YchH and SmpA

YfgM, YchH and SmpA are all small membrane proteins of which there is little known. They are conserved in *Salmonella, E. coli, Shigella* and *Yersinia* species and appear to be up-regulated in response to σ^E from our microarray data. In particular, SmpA appears to be highly up-regulated in array studies. SmpA was very recently identified as being an additional, nonessential component of the YaeT complex which is important for the assembly of outer membrane proteins (346). YfgM is part of the *yfg-eng* locus known to be involved in cell invasion and the colonisation of chicks in *Salmonella enteritidis* (11). Further characterisation of these small proteins would

help to understand their role in the RpoE response and also hopefully their wider role in bacteria.

PqiA

pqiA and *pqiB* are both paraquat inducible genes that show up-regulation in our over-expressing RpoE microarray. Previous studies within our laboratory have shown σ^E to be important in the defence against superoxides including paraquat (167) and as such I chose to characterise these genes and investigate the potential role of paraquat in the RpoE ESR.

The first step in the characterisation of these putative RpoE regulated genes was the construction of mutants using Red mutagenesis to replace the gene of interest with a kanamycin (or other antibiotic) resistance cassette as described in method 2.7.2.

This process of PCR mediated gene replacement was refined within our lab for the efficient mutagenesis of various *S.* Typhimurium genes (302) using hyper-recombinogenic strains of *S.* Typhimurium (331). These *Salmonella* strains (GVB1327 and GVB1335, Table two) allow rapid recombination by utilising the bacteriophage λ RED system with λ Red recombinase being synthesised from an arabinose inducible promoter. This system is a straight-forward, highly efficient method allowing for the rapid mutagenesis of many genes. Oligonucleotides ddgREDFor2, ddgREDrev, slyBREDFor, slyBREDRev, NlpBREDRev, NlpBREDFor, smpAREDFor, smpAREDRev, IbpAREDfor, IbpAREDRev, IbpBREDfor, IbpBREDRev, ychHREDFor, ychHREDRev, yfgMREDFor, yfgMREDRev, pqiAREDFor and pqiAREDRev as shown in table four were used in the mutagenesis of *ddg, slyB, nlpB, smpA, ibpA, ibpB, ychH, yfgM* and *pqiA*

respectively. External oligonucleotides ddgextF, ddgextR, slyBextF, slyBextR, nlpBextR, nlpBextFor, smpAextF, smpAextR, ibpAextF, ibpAextR, ibpBForEXT, ibpBRevEXT, ychHForEXT, ychHRevEXT, yfgMForEXT yfgMRevEXT, pqiAextForEXT and pqiAextRev2 (table four) were used for PCR verification of the genotype of each mutant.

PCR verified colonies produced from λ Red mutagenesis were cured at 30ºC to remove the temperature sensitive plasmid and transduced to a wild type SL1344 *S.* Typhimurium strain via p22 transduction. A selection of colonies were picked for PCR verification of mutagenesis status. Figure 3.4 shows the amplification bands achieved for several of these mutants compared to the WT parent strain.

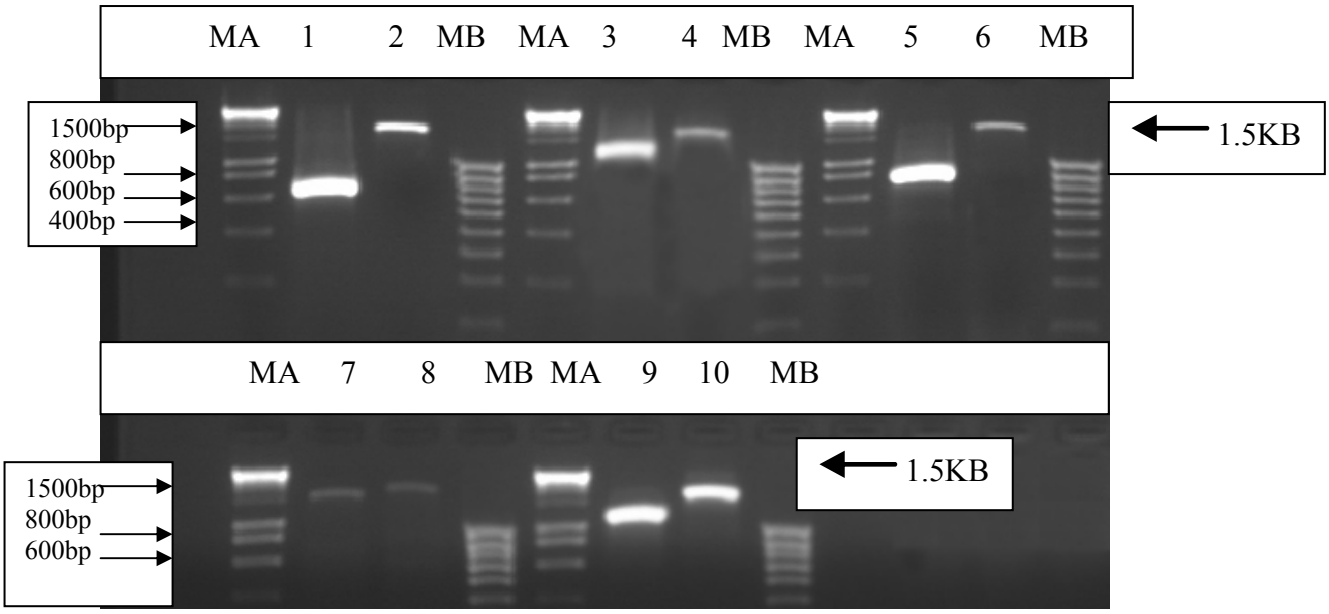


Figure 3.4 - PCR used to check genotype of S. Typhimurium mutants *slyB, nlpB, yfgM, pqiA* and *ddg*

PCR verification was performed as in 2.7.2, The mutant size of slyB (2), nlpB (4), yfgM (6) and pqiA (10) corresponds with the 1.5Kb kanamycin cassette inserted into the wild type copy of the appropriate gene as determined by Hyperladder I DNA ladder (MA) and Hyperladder IV DNA ladder (MB). WT slyB (1), WT nlpB (3), WT yfgM (5), WT pqiA (7) and WT ddg (9) produced amplicons at approximately 460bp, 1Kb, 600bp, 1.2Kb and 900bp respectively, corresponding with the expected WT amplicon size.

3.5 *In vitro* analysis of putative RpoE regulated *S.* Typhimurium mutants

Following construction of SL1344 *slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg* mutants, several phenotypic assays were set-up to examine the behaviour of these strains compared to the wild type SL1344 *S.* Typhimurium. As very little is known about the group of putatively RpoE regulated genes chosen in this study, initial characterisation work involved looking at the growth kinetics of these bacterial strains at various temperatures and using different chemical compounds which have been previously shown to be involved in the extracytoplasmic stress response. With regard to this, assays were chosen which have previously been shown to affect an SL1344 *rpoE* mutant.

As such, preliminary studies were set up to examine growth of our mutants at 42ºC and 46ºC. Several different protocols were tested to investigate growth at varying temperatures with the Bioscreen C machine proving to be the most accurate. This machine permits the testing of many strains simultaneously, and allows for many replicates to be tested, adding to the statistical significance of the assay.

Figures 3.5 and 3.6 show the growth of different *Salmonella* strains in liquid culture at 42ºC. As can be seen from Figures 3.5 and 3.6, the *rpoE* mutant strain exhibited a longer lag phase as shown previously(167, 406); however the final optical density following 16 hour growth was not significantly reduced compared to WT SL1344. In Figure 3.5, mutant SL1344 *slyB, nlpB, yfgM, pqiA,* and *ddg* strains show no significant change in either lag phase or log phase growth. Similarly, in Figure 3.6, mutant SL1344 *smpA, ibpA, ibpB* and *ychH* strains show no significant change in either lag phase or log phase growth.

Figures 3.7 and 3.8 show the growth of different *Salmonella* strains in liquid culture at 46ºC. At this extreme temperature a much more dramatic difference can be observed between SL1344 WT and the *rpoE* mutant strain. At this temperature, the SL1344 *rpoE* strain struggles to grow, and exhibits a much reduced growth rate than the SL1344 WT strain. Most other mutant strains tested in Figures 3.7 and 3.8 show no defect in growth compared to SL1344 WT. SL1344 *smpA* shows a slight overall reduction in growth compared to SL1344 WT, however this is not significantly different (P = 0.5396, T-test)

Similar growth kinetic results were achieved when studying the growth of all the above bacteria in liquid cultures incubated in either a shaking water-bath or static temperature incubators, however data obtained from the Bioscreen C machine appeared to be more robust and consistent, and as such was chosen as the method of choice for our growth kinetic studies.

Growth kinetics of the above bacterial strains were then examined in the presence of various concentrations of hydrogen peroxide and ethanol as the *rpoE* mutant exhibits increased sensitivity to these compounds (167).

The Bioscreen C machine was used as described in method 2.8.1 to study the effect of H_2O_2 and ethanol.

Concentrations of 0.2, 0.4, 0.6 and 0.8mM H_2O_2 were tested, with Figures 3.9 and 3.10 showing the growth kinetics for various strains with 0.2mM H_2O_2.

From these results it can be seen that there is no observable difference between SL1344 WT and the mutant strains tested (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA* and *ddg*). The *rpoE* mutant however shows slight inhibition compared to SL1344 WT as expected. Similar results were achieved at all concentrations of hydrogen peroxide tested. 2%, 3% and 4% ethanol were tested and again mutation of

none of the genes tested (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA* and *ddg*) induced any observable difference in growth compared to SL1344 WT, however SL1344 *rpoE* showed an observable reduction in growth compared to SL1344 WT at all concentrations of ethanol tested. Although there was an apparent difference in growth between SL1344 *rpoE* and SL1344 WT, the *rpoE* strain still managed to grow and survive at these concentrations of ethanol, although at a reduced rate. All strains were finally tested in liquid media with the presence of 5% ethanol however this proved toxic to all strains. To complement these growth kinetic studies, disc diffusion assays were performed as in method 2.8.2, using various antibiotic discs.

Table seven lists the antibiotics tested against our 9 putatively RpoE regulated genes and their presumptive mode of action.

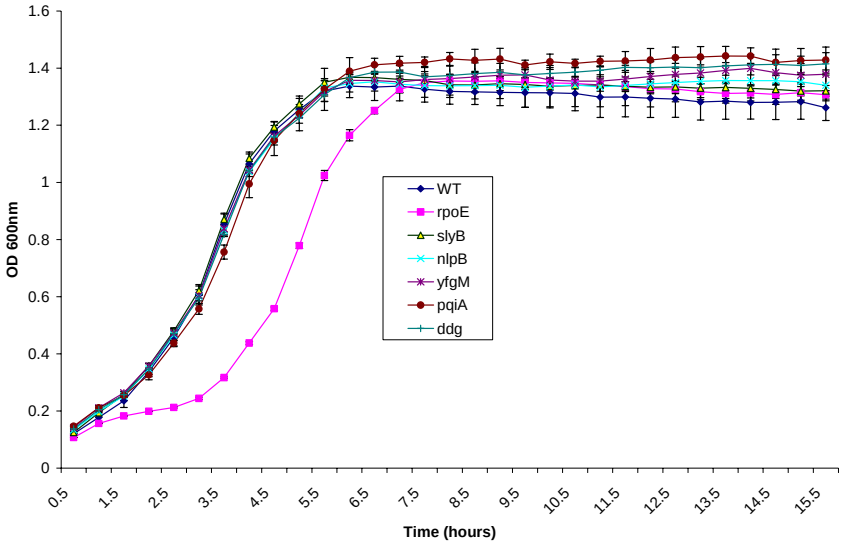


Figure 3.5 - Effect of *slyB, nlpB, yfgM, pqiA* and *ddg* mutations on the growth of *S.* Typhimurium in liquid media.
The *slyB, nlpB, yfgM, pqiA* and *ddg* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture at 42ºC using a Bioscreen machine. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

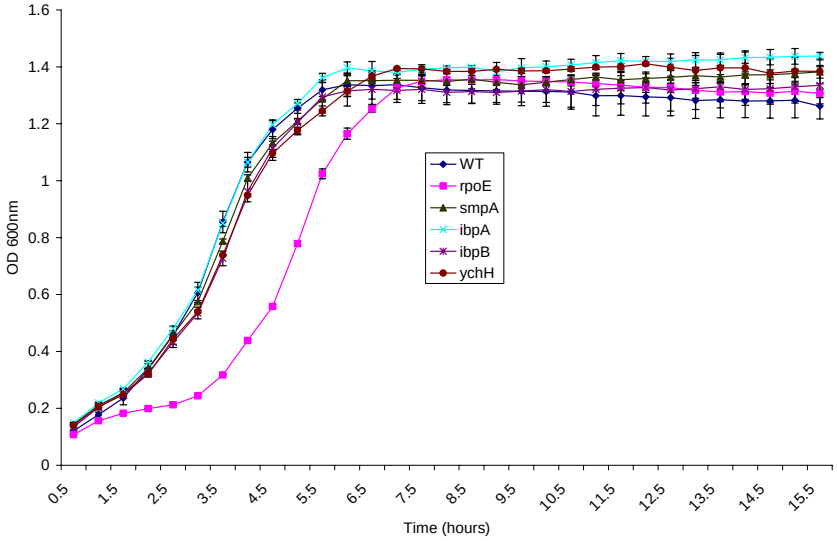


Figure 3.6 - Effect of *smpA, ibpA, ibpB* and *ychH* mutations on the growth of *S.* Typhimurium in liquid media.
The *smpA, ibpA, ibpB* and *ychH* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture at 42ºC using a Bioscreen machine. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

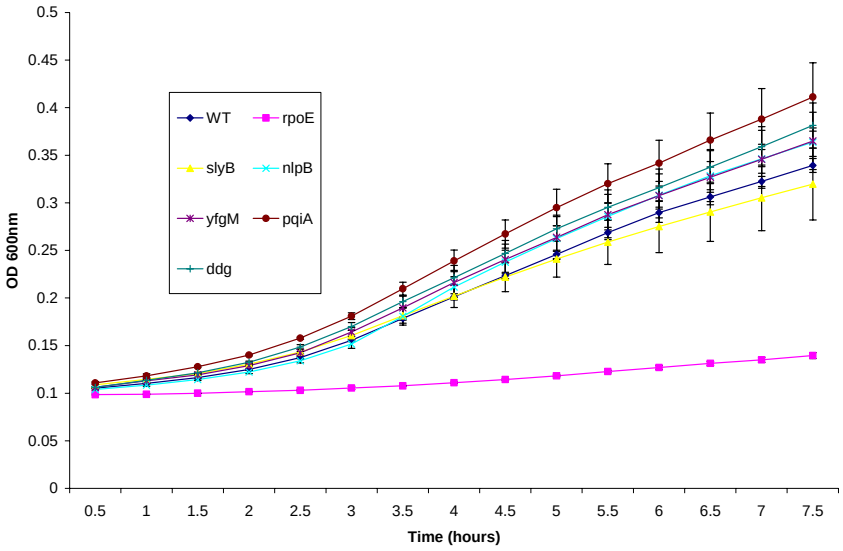


Figure 3.7 - Effect of *slyB, yfgM, ddg, nlpB* and *pqiA* mutations on the growth of *S.* Typhimurium in liquid media.
The *slyB, yfgM, ddg, nlpB* and *pqiA* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture at 46ºC using a Bioscreen machine. Growth was monitored for 8 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

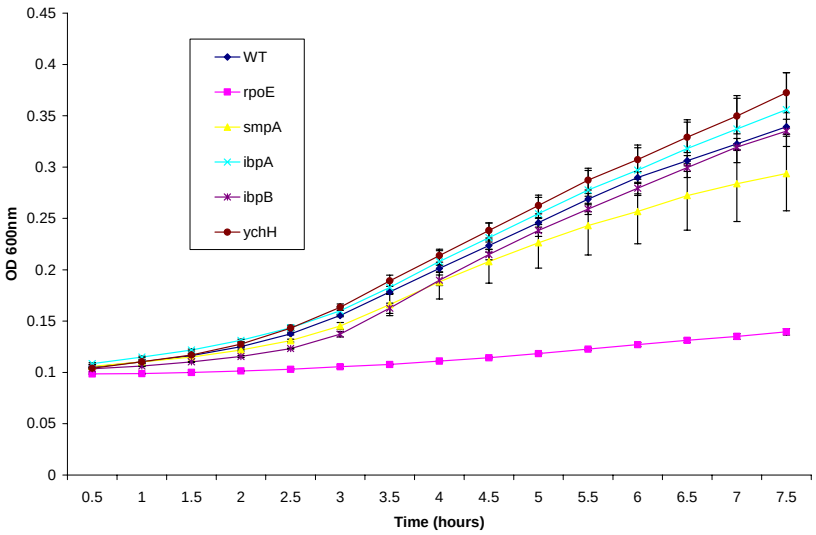


Figure 3.8 - Effect of *smpA, ibpA, ibpB* and *ychH* mutations on the growth of *S.* Typhimurium in liquid media at 46^{o}C
The *smpA, ibpA, ibpB* and *ychH* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture at 46ºC using a Bioscreen machine. Growth was monitored for 8 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

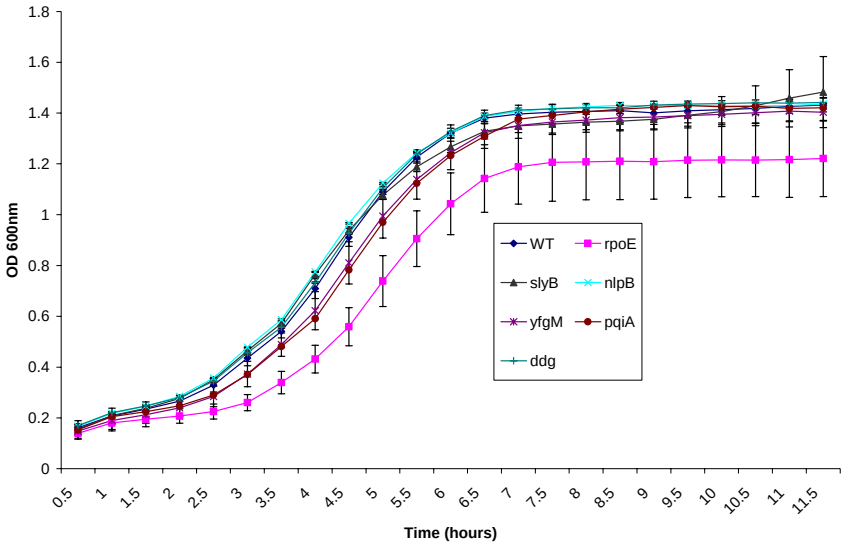


Figure 3.9 - Effect of *slyB, yfgM, nlpB, pqiA* and *ddg* mutations on the growth of *S.* Typhimurium in liquid media supplemented with 0.2mM hydrogen peroxide
The *slyB, yfgM, nlpB, pqiA* and *ddg* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture supplemented with 0.2mM hydrogen peroxide at 37º, using a Bioscreen machine. Growth was monitored for 12 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

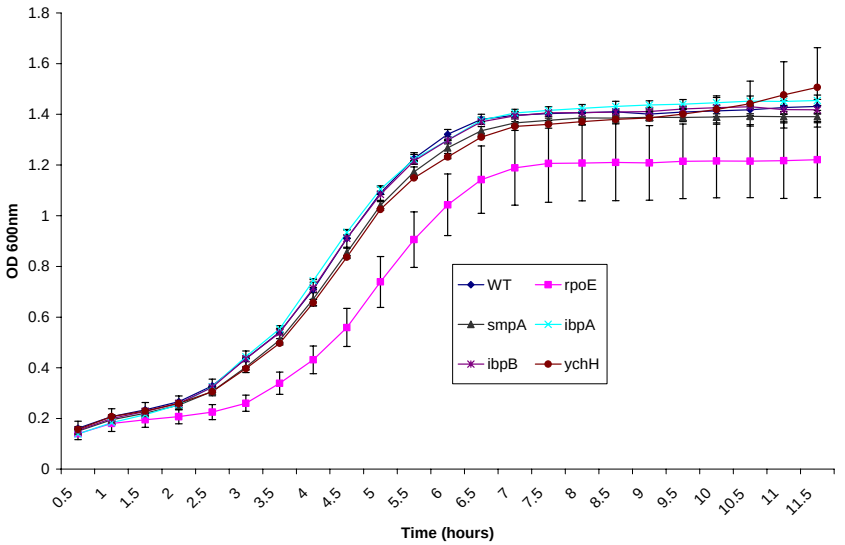


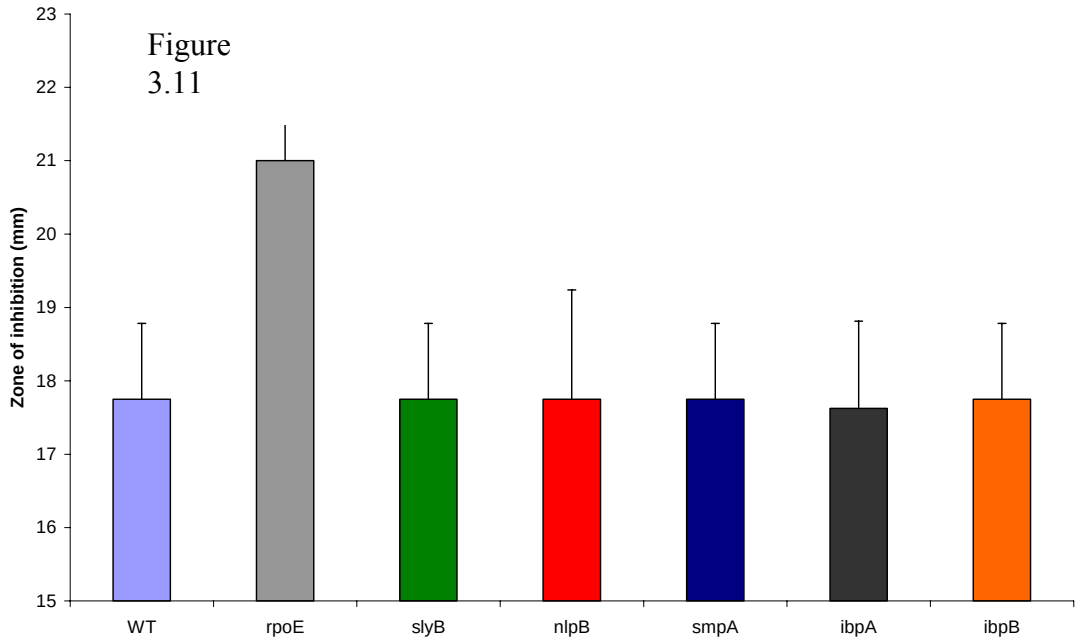
Figure 3.10 - Effect of *smpA, ibpA, ibpB* and *ychH* mutations on the growth of *S.* Typhimurium in liquid media supplemented with 0.2mM hydrogen peroxide
The *smpA, ibpA, ibpB* and *ychH* mutations were compared with the WT strain and the *rpoE* mutant for their ability to grow in culture supplemented with 0.2mM hydrogen peroxide at 37º, using a Bioscreen machine. Growth was monitored for 12 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

Table seven. Antibiotics listed in this table were selected for sensitivity testing against *slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA, ddg* and WT SL1344.

Antibiotic (Class)	Action
Chloramphenicol	Protein synthesis inhibitor
Gentamicin (aminoglycoside)	Protein synthesis inhibitor
Erythromycin (macrolide)	Protein synthesis inhibitor
Tetracycline (tetracycline)	Protein synthesis inhibitor
Bacitracin	Cell-wall biosynthesis inhibitor
Ciprofloxacin (fluoroquinolone)	Gyrase inhibitor
Polymyxin B (cationic antimicrobial peptide)	Binds to lipid A core of LPS

The antibiotics shown above were selected to allow us to study the effect of different antibiotic actions. As mentioned previously, an *S.* Typhimurium *rpoE* mutant has increased sensitivity to polymyxin B, at a concentration of 300 units(167, 406). In this study however, there was no observable difference in polymyxin B sensitivity between SL1344 WT and any of the 9 mutant strains (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg*) when tested with polymyxin B as shown in Figure 3.11 and 3.12. In fact, there was no significant difference in the antibiotic sensitivies of SL1344 WT and *slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* or *ddg* for any of the antibiotics tested.

Following disc diffusion assays, inhibition assays were set-up as detailed in 2.8.3 to investigate the effect of membrane-damaging agents on the behaviour of all 9 of our



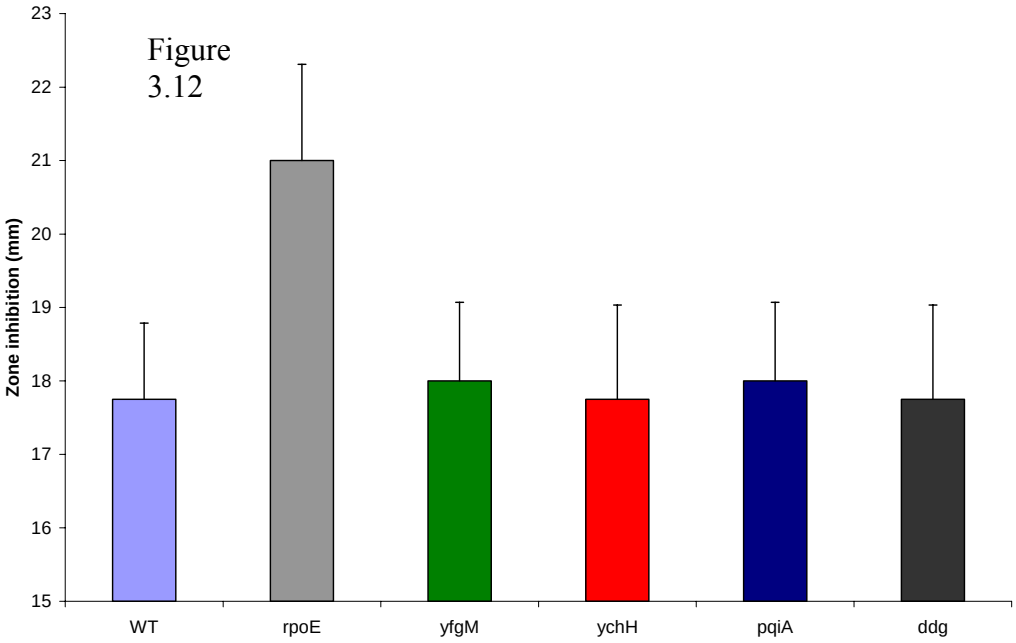


Figure 3.11 and Figure 3.12 – Sensitivity of *S.* Typhimurium mutants to 300U polymyxin B.
All mutant and WT strains were tested for their sensitivity to 300U of the antimicrobial peptide polymyxin B by a disc diffusion assay. Bars represent the mean diameters of the zone of inhibition (mm) of 8 replicates whilst the error bars represent the standard deviation of the mean. Figure 3.11 shows SL1344 WT, *rpoE, slyB, nlpB, smpA, ibpA* and *ibpB,* and 3.12 shows SL1344 WT, *rpoE, yfgM, ychH, pqiA* and *ddg*.

mutant strains of *S.* Typhimurium. SDS, sodium cholate (bile acid) and CCCP (a protonophore that disrupts the proton motive force) were tested. SDS was tested in liquid media at concentrations of 1 and 2 % however there was no observable difference between all 9 mutant strains tested (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg)* compared to SL1344 WT. CCCP was tested at concentrations of 8 and 16μg/ml and as shown in Figure 3.13, SL1344 *smpA* was significantly more sensitive than WT to CCCP (unpaired T-test, $P< 0.0001$) at a concentration of 8μg/ml however all other strains tested showed no significant difference. Concentrations of CCCP at 16μg/ml proved toxic to all strains tested.

Sodium cholate was tested at concentrations of 1 and 5 %, and interestingly, in all strains tested there appeared to be an increase in growth when media was supplemented with 1% sodium cholate, compared to media containing no sodium cholate, however in media supplemented with 5% sodium cholate, there was a reduction in growth in all strains tested, compared to media containing no sodium cholate. In media containing 5% sodium cholate the *smpA* mutant was significantly more sensitive than WT ($P<0.001$, T-test) as shown in Figure 3.14, however there was no observable difference in all other strains tested.

3.6 *In vivo* analysis of RpoE regulated *S.* Typhimurium mutants

To investigate any role of virulence *in vivo* from our list of 9 mutants, competition assays were established as detailed in 2.8.5.1. Briefly, mice were given an inoculum containing 10^3 CFU of both wild type *S.* Typhimurium and one of the mutant strains under investigation (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* or *ddg*) by IP injection. A CI of ~1.0 indicates that the strains compete equally well. Statistical

significance of the CI was measured using a two tailed unpaired t test. Table 8 highlights the average CI obtained for each of these mutant strains.

From this data it appeared that the SL1344 *ychH* strain appeared to be more virulent than WT.

Table 8- Effect of *slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg* on S. Typhimurium virulence. Groups of 5 mice were challenged with a mixed dose of ~10^3 CFU WT SL1344 and the appropriate mutant strain via IP injection. * indicates statistical significance using student T-test.

Strain	Spleen CI	Liver CI	Mean CI
ibpAΔ	0.87	1.18	1.03
ibpBΔ	0.65	0.51	0.58
nlpBΔ	0.97	0.98	0.98
smpAΔ * p= 0.0014	0.09	0.06	0.08
slyBΔ	0.52	0.64	0.58
ddgΔ	1.32	2.45	1.89
pqiAΔ	1.14	1.88	1.51
yfgMΔ	0.93	2.93	1.93
ychHΔ	3.2	3.4	3.3

From the list of 9 mutant strains (*slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg*), it appeared that only SL1344 *smpA* showed any observable difference to WT under the parameters tested above. As such, SL1344 *smpA* was selected for further study.

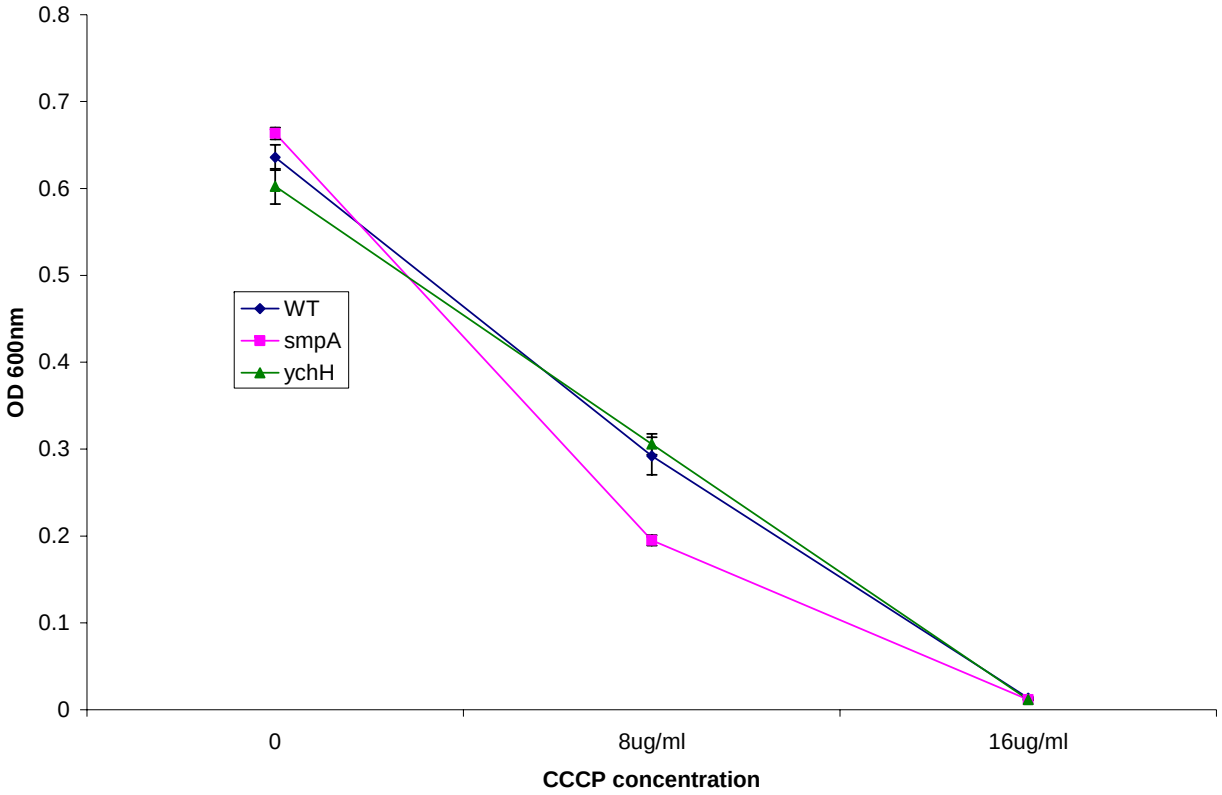


Figure 3.13 - Effect of *smpA*,and *ychH* mutations on the growth of *S.* Typhimurium in liquid media supplemented with CCCP
The *smpA,* and *ychH* mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 8, and 16µg/ml CCCP at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

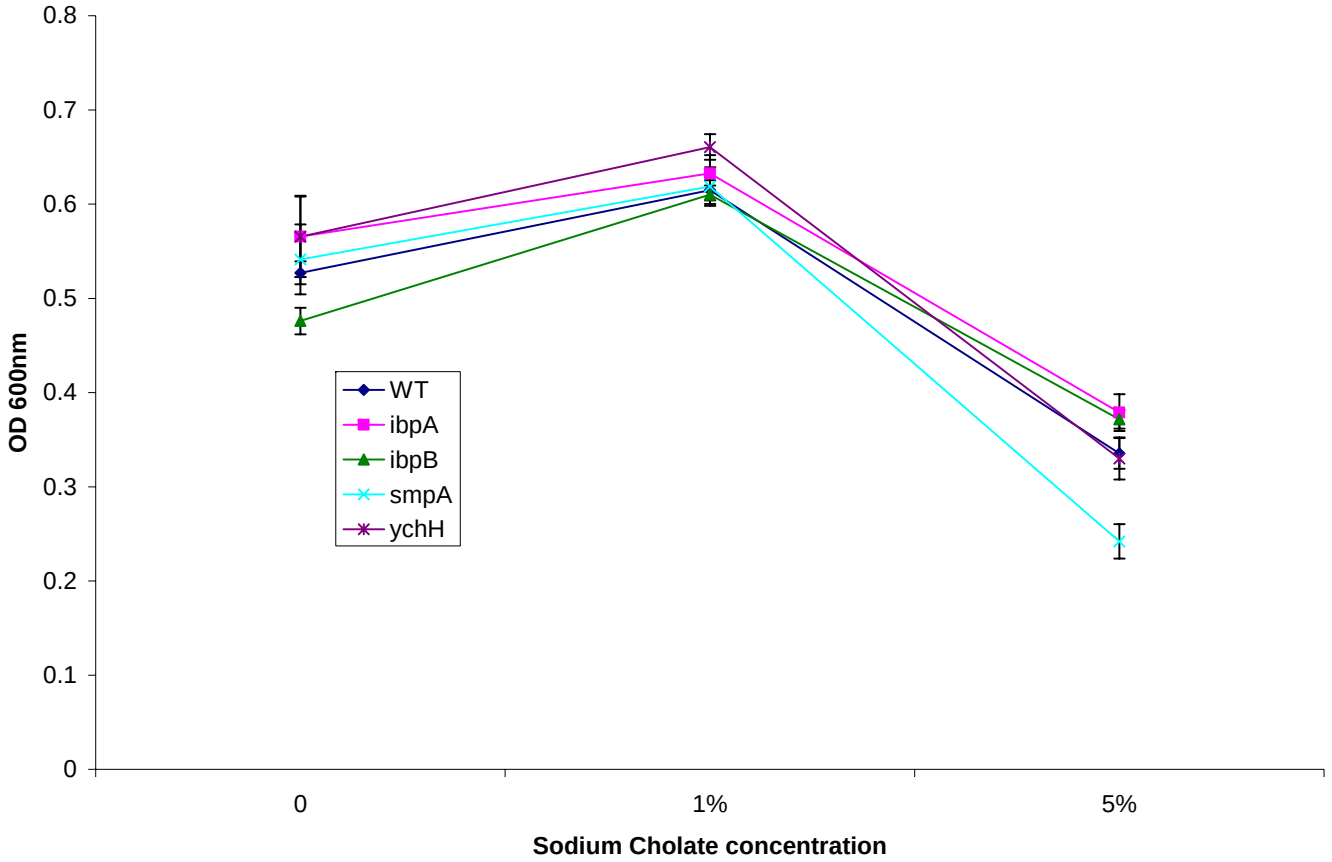


Figure 3.14 - Effect of *ibpAΔ, ibpBΔ*, *smpAΔ*,and *ychHΔ* mutations on the growth of *S.* Typhimurium in liquid media supplemented with sodium cholate The *ibpAΔ, ibpBΔ smpA,* and *ychH* mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 1, and 5% sodium cholate at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

3.7 Extended smpA *in vivo* analysis

3.7.1 Invasion and survival of SL1344 *smpA* in a macrophage like cell-line.

To further characterise SL1344 *smpA*, cell-culture assays were established to investigate the invasion of *S.* Typhimurium strains in the macrophage like cell-line Raw 264.7. Figure 3.15 shows the respective abilities of SL1344 WT, *smpA* and *rpoE* to invade and persist within the cell line. From this data, we can see that SL1344 *smpA* and *rpoE* have a reduced ability to invade and persist in this cell-line ($P<0.005$, ANOVA, considered extremely significant).

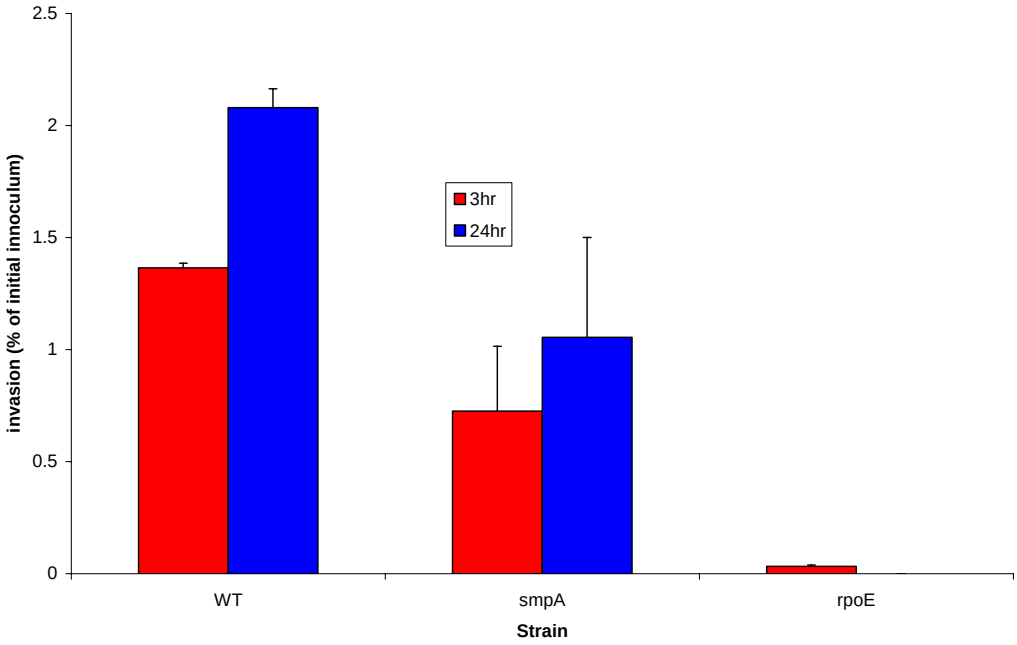


Figure 3.15 - The effect of a *smpA* mutation on *S.* Typhimurium invasion and survival in macrophages.
Bacteria at a multiplicity of infection of ~ 1:1 were incubated with the murine macrophage cell line RAW 264.7. The graphs show the number of viable bacteria (as a % of the initial inoculum) inside the macrophage at 3 h (red bars) and 24 h (blue bars) after infection. Each bar represents the mean from triplicate experiments and the error bar indicates the SD.

3.7.2 Analysis of *smpA* virulence following oral infection of mice

Groups of 5 BALB/c mice were infected with single doses of either SL1344 WT or *smpA* bacteria via the oral route, to mimic the natural route of infection. One group of mice was infected with 1 x 10^7 CFU SL1344 *smpA* bacteria, and a second group of mice were infected with 1 x 10^5 CFU WT bacteria, both by oral gavage. The group infected with WT bacteria were given a lower dose as bacterial numbers above this threshold cause rapid death. Mice were culled 7 days post-infection and counts were performed on livers, spleens, mesenteric lymph nodes (MLN) and Peyers patches as detailed in 2.8.5.2. As can be seen in Figure 3.16, SL1344 *smpA*, although infected at a much higher concentration, cannot replicate to the same degree as WT SL1344 in either the spleen or the liver of the mice. In the MLN or Peyers patches, the level of replication is approximately the same however mice infected with WT were subjected to a much lower concentration.

As such SL1344 *smpA* has significantly reduced ability to infect and replicate within a murine model compared to SL1344 WT.

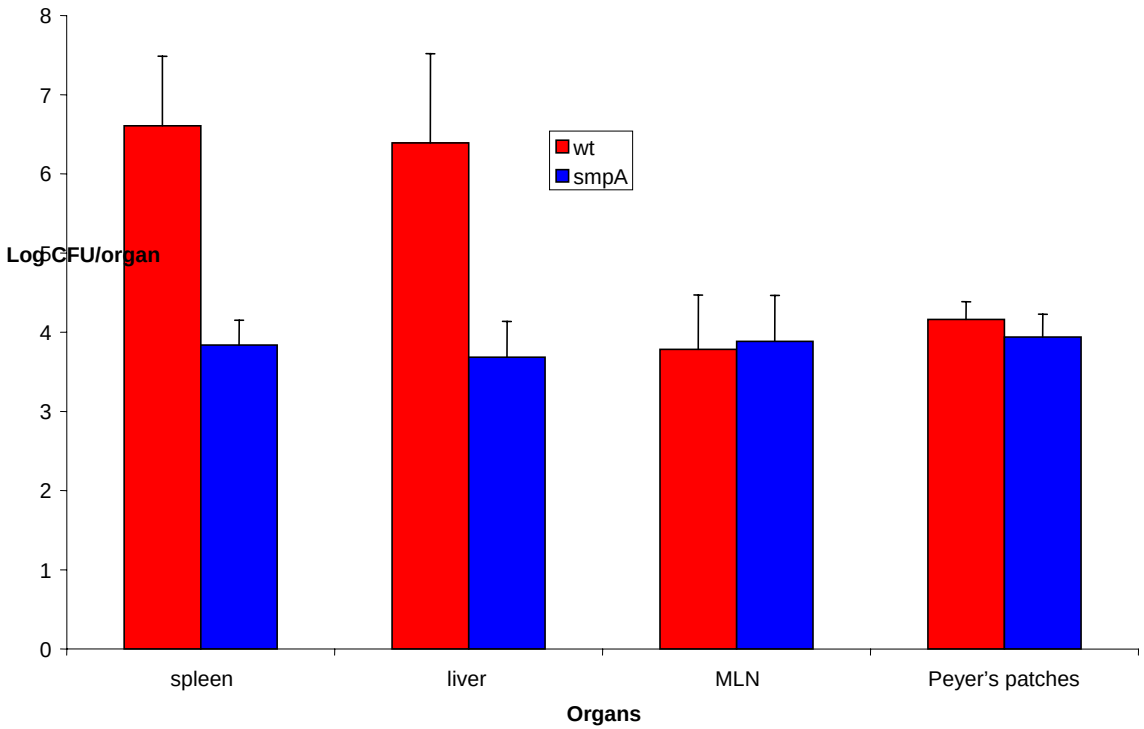


Figure 3.16 - Oral Infection of BALB/c mice with wild type *S.* Typhimurium and an isogenic *smpA* mutant.
Single doses of $1x10^5$ CFU WT bacteria or 1×10^7 CFU SL1344 *smpA* were administered by oral gavage and CFU/organ counts performed 7 days post infection. Data presented is a mean of 5 mice and error bars represent the standard deviation of the mean.

3.8 Discussion

My studies began with the identification and characterisation of a number of *S.* Typhimurium RpoE regulon members. In addition to helping to understand how *Salmonella* can survive environmental stress it was thought that study of S. Typhimurium RpoE regulon members may identify genes that could be used in future vaccine development studies. Loss of *rpoE* itself causes attenuation to such a great degree that it is ineffective as a vaccine, probably because it is cleared very quickly from the host before a significant immune response can be established (167). However, it is possible that loss of individual RpoE regulon members may cause attenuation to a lesser degree and as such be possible contenders in vaccine development studies as is the case for the RpoE regulated genes *htrA* and *surA* (49, 364).

Previous studies by ourselves and our collaborators has identified a large number of presumptive *S.* Typhimurium RpoE regulated genes by both microarray analysis and a 2-plasmid promoter search (331, 349). In the case of the 2-plasmid screen where the presence of an RpoE dependent promoter was confirmed, 62 genes were shown to be members of the RpoE regulon (349). Some of the same but also additional RpoE regulated genes (6 genes) were identified by a 2-plasmid screen for *rpoE* controlled genes in *E.coli* (323). It is likely that some of these additional genes are also RpoE regulated in *S.* Typhimurium as they are upregulated in *S.* Typhimurium in our array data and for one gene, *smpA*, it was definitely demonstrated to be RpoE regulated in *S.* Typhimurium (213).

A similar microarray study to ours using *E.coli* over expressing RpoE identified ~ 200 genes that were upregulated when RpoE was over expressed (187). This and our own studies indicate that there are many RpoE regulated genes in *S.* Typhimurium and *E.coli* and a significant number have no known function.

Our microarray data was based on the study of an over-expressing RpoE strain compared to wild type, rather than an *rpoE* mutant as the quality of RNA obtained from these strains was far superior (333) and also *S.* Typhimurium *rpoE* mutants are stressed and other stress response systems are up regulated, in some cases highly upregulated, for example the Cpx system (165) which can complicate the microarray analysis. However for further clarification of the *S.* Typhimurium RpoE regulon, it would be useful to repeat our microarray experiment using good quality RNA from an *rpoE* mutant.

Following a promoter consensus search for RpoE dependent promoters and RT-PCR analysis on a few of these genes, several putatively RpoE regulated genes *(slyB, nlpB, smpA, ibpA, ibpB, yfgM, ychH, pqiA,* and *ddg)* were selected for further characterisation. All 9 of the genes selected were up-regulated in our microarray experiment. Improvements on this part of my study would be to expand on the RT-PCR performed by testing the expression profiles of all 9 genes selected and also examining the expression of the genes in an *rpoE* mutant strain. We could also confirm the that the putative *rpoE* dependent promoter is functional and if there are any other promoters upstream of the genes by performing S1 mapping or primer extension analysis, for example see (213). However, so far this has been performed

by our collaborator Jan Kormanec (Slovakia) as he is very experienced with these methods and it would seem sensible for him to perform such analysis.

The genes studied further were selected for a number of reasons. For example, that they might be involved in virulence, such as *ddg* (*lpxP*) which modifies LPS and modification of *S.* Typhimurium is known to be important *in vivo* (248). The *pqiA* gene would also fall into this category. Cell envelope proteins are often involved in interacting with the host and/or carry out important roles for pathogens *in vivo*, such as HtrA and SurA. A number of the genes encode putative envelope proteins such *nlpB, smpA, yfgM,* and *ychH*. The genes *ibpA* and *ibpB* were selected for further study as they are thought to be homologues of *STM1250* and *STM1251* which have been previously identified as RpoE regulated genes in *S*. Typhimurium (331).

Mutagenesis of each of these genes was successfully achieved which itself implies that none of these genes were essential to the survival of *S.* Typhimurium SL1344 under normal conditions. This might be expected as RpoE itself is not essential for *S.* Typhimurium (167), contrary to what was found in *E. coli* (74, 167). It should not be assumed however that all genes identified as up-regulated in our microarray experiment or RpoE regulated genes in general are non-essential as *yaeT* is one such up-regulated gene which has been shown to be essential for *S*. Typhimurium (195). Jan Kormanec has confirmed that *yaeT* is RpoE regulated (personal communication) and both his and our group have been unable to construct a *S.* Typhimurium *yaeT* mutant supporting the suggestion that it is an essential gene. YaeT will be discussed further below.

I examined if mutation of the different genes affected the ability of the *S.* Typhimurium to survive different stresses. The first of these was growth temperature. Unlike its *E.coli* counterparts, *S.* Typhimurium *rpoE* mutants grow relatively normally at 42^{o}C (167). However, I was able to show here that at 46^{o}C growth of *S.* Typhimurium *rpoE* was almost completely inhibited. Therefore this temperature can be used to screen for RpoE regulated genes necessary for growth at high temperature. Apart from *smpA* none of the genes was important for resistance to any of the stresses tested or was important for salmonella virulence. A relatively small number of toxic substances or harsh environments were tested and use of a wider range of toxic substances or other environments may have revealed phenotypes for the other mutants. It is possible that adverse phenotypes were not seen for the mutants tested because of genetic redundancy, that is, the loss of one gene function is compensated by the presence of another gene. A function for the first gene in this case would only be revealed if the second genes was also absent. There is certainly a precedent for this with RpoE regulated genes. An effect of mutation in *fkpA* on *S.* Typhimurium virulence was only seen if the strain was also lacking *surA* (166). *S.* Typhimurium posses a homologue of HtrA called DegQ (or HhoA), absence of DegQ has no obvious effect on *S.* Typhimurium but a *S.* Typhimurium *htrA degQ* mutant is more attenuated than a *S.* Typhimurium *htrA* mutant (101).

The *in vivo* IP model used examines systemic infection over a short period (3 days). The effect of loss of any of the other genes selected might only be observed at a later time and as such future studies could focus on the development of an *in vivo* assay to monitor infection over a longer period of time. If any of the mutations affected the ability of *S.* Typhimurium to infect the intestinal tract but not growth at systemic sites

then this would not be revealed from the IP infection. So for particular genes if we wanted to fully investigate their role in *S.* Typhimurium pathogenesis oral infection studies should also be performed, however, available evidence indicates that in *S.* Typhimurium RpoE regulated genes are much more important for systemic infection than enteric infection (167, 331, 364).

The *S.* Typhimurium *smpA* mutant was the only one which showed significant attenuation in an IP model. Interestingly, a mutation in *ychH* appeared to make this strain more virulent than the WT. It could be that this is a false result due perhaps to the small number of replicates tested in our I.P studies, or perhaps an error at either the dosing stage or bacterial counting stage and it is hoped that these studies could be repeated for further clarification.

At this point in my studies *smpA* was the only gene from the 9 selected that appeared to have detectable phenotypes different from the WT strain. These are: increased sensitivity to sodium cholate and CCCP, defective infection of macrophage-like cells, and attenuation in a murine model of infection. Following the demonstration that the *smpA* mutant was attenuated in the IP competition assay I wished to investigate the effect of the *smpA* mutation on *S.* Typhimurium pathogenesis more fully. In particular I wanted to investigate if SmpA is important for *S.* Typhimurium pathogenesis via the natural oral route of infection. For these studies mice were infected singly with either the WT or *smpA* strains. The two groups were given different doses of the two organisms. The mice were given a higher dose of the *smpA* mutant than the mice that received the WT strain. The dose the *smpA* group received (1×10^7) was considerably higher than the oral LD50 of the WT strain SL1344 (167). The reason such high

doses were given was that because of the attenuation of the *smpA* mutant there was a concern that there would be little or no bacteria present in the tissues at the time of harvest. In contrast, if the equivalent dose of the *smpA* mutant were given to the WT mouse group then the mice were likely to die before the end of the experiment. Therefore, the counts were normalised for the infecting dose. There is debate as to whether this is the correct way to perform this experiment but note that the results have been peer reviewed and published (213) and also that this is the accepted procedure in the commonly used competition assay. This is the first time that a role in pathogenesis has been demonstrated for SmpA in any organism.

E.coli and *P. aeruginosa* mutants that lack SmpA (called OmlA in *P. aeruginosa*) have been created (278, 346). For both species the loss of SmpA has a greater effect on the sensitivity of the organisms to detergents and other agents than it does in *S.* Typhimurium (278, 346).

SmpA was recently identified as a member of the YaeT complex responsible for assembly of OMP's. The YaeT complex is composed of the OMP YaeT and the lipoproteins SmpA, NlpB, YfiO and YfgL (33). In *E.coli* both YaeT and YfiO are essential whereas the other components are not (33, 229, 346). As mentioned above YaeT is essential in *S.* Typhimurium (195), however our group has constructed a *S.* Typhimurium *yfiO* mutant (MR personal communication). Interestingly, my studies show that components of the YaeT complex differ in their importance to *S.* Typhimurium *in vivo* as *smpA* mutants are attenuated whereas *nlpB* mutants are not. Therefore it would be very interesting to see if the other non-essential components of the YaeT complex, YfiO and YfgL, have a role in *S.* Typhimurium pathogenesis. If

any of them do it would be interesting to see what the effect is of combining mutations in the genes of the YaeT complex. For example, are the strains with multiple mutations more attenuated than single mutants and are they useful as live vaccines. Finally the reason for the attenuation of S. Typhimurium *smpA* (and possibly other YaeT complex mutants) could be studied. For instance do the mutations affect the production of OMPs or surface structures important for virulence, for example, are there effects on the type III secretion systems?

Chapter Four - Characterisation of the Phage Shock response, the fourth ESR.

4.1 Introduction

The Phage shock proteins (psp) were first identified as stress proteins of *E. coli* (38) produced at high concentrations during filamentous phage infection. The psp response is now known to be a very important extracytoplasmic stress response, conserved in many bacteria, including *S.* Typhimurium (69, 261).

Characterisation of the psp in *E. coli* (39) discovered it was produced maximally when under extreme stress conditions and it is known to ensure survival of *E. coli* in late stationary phase at alkaline pH, and protect the cell against a disrupted proton-motive force (37, 193).

Initially the *psp* operon was thought to consist of genes *pspABCDE*, with expression controlled by the transcriptional activator pspF. However, another gene, *pspG* has recently been identified in *Y. enterocolitica* (131), shortly followed by its identification in *E. coli* (222) however it is not part of the main *psp* operon. *pspG* is now thought to exist in other enteric bacteria including *Y. pestis*, *Shigella* species, and also *S.* Typhimurium (previously known as *yjbO*) (131).

Figure 4.1 shows the operon structure of the main *psp* genes and a working model for the psp response systems in *S.* Typhimurium is shown in Figure 4.2.

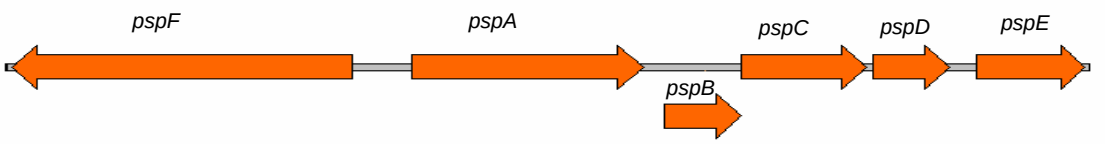


Figure 4.1 - The *psp* Operon in *S.* Typhimurium.

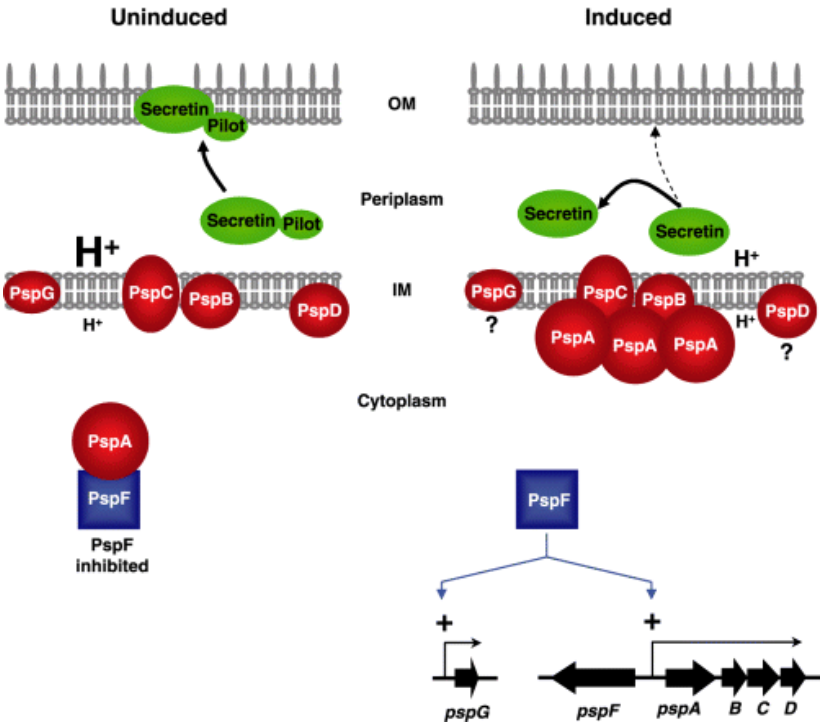


Figure 4.2 - Working model for the psp response systems of *S.* Typhimurium. In the uninduced situation, PspA binds to PspF and prevents it from activating transcription. Several conditions are known to induce the Psp response, many of which may reduce the proton-motive force. The mislocalization of a secretin protein, which can be artificially caused by removal of its chaperone-like pilot protein, is a highly specific inducing signal. It is not known whether secretin mislocalization also reduces the PMF, or activates via an independent mechanism. One or both of the cytoplasmic membrane proteins PspB and PspC may sense an inducing signal and then bind PspA. This releases PspF, which activates the *pspA* and *pspG* promoters. Upon induction, PspA becomes the most abundant Psp protein and may predominantly associate with the cytoplasmic membrane, where it serves a physiological function such as helping to maintain PMF. PspD and PspG do not play a role in the signal transduction pathway and their functions are unknown. IM, inner membrane; OM, outer membrane. Adapted from (67).

It is thought that under normal conditions PspA binds to PspF, preventing Psp transcription (87) and following an inducing signal such as disrupted proton motive force (193), or secretin mislocalisation, PspB and/or PspC interact with PspA, releasing PspF to initiate Psp transcription. At this stage the precise roles of pspD and pspG are not known.

As mentioned in chapter 3, the *psp* genes were one class of genes identified within our laboratory during studies to characterise the RpoE regulon in *S.* Typhimurium. Briefly, a micro-array experiment was performed by comparing gene expression of *S.* Typhimurium strains which over-expressed *rpoE* against the wild type, in collaboration with a group at the Institute of Food Research (331). The same group performed similar micro-array experiments looking at the regulation of *S.* Typhimurium genes in macrophages, which lead to the identification of candidate genes regulated under macrophage infection (95). Overlapping the two micro-array experiments identified several genes, of which the *psp* (phage shock protein) genes were the most highly up regulated. In addition to this, research has shown the *pspC* mutant in *Y. enterocolitica* to be severely attenuated for virulence (68). Further, the *pspC* gene of *Y. enterocolitica* was found to be necessary for normal growth when the *Ysc* TTSS was expressed (69). This research was the first indication of Psp proteins being involved in the regulation of genes outside of the Psp locus itself. As such, part of my study was to investigate the role of these phage shock proteins in relation to virulence and regulation by *rpoE*. Null mutants for the whole psp operon were constructed in *S.* Typhimurium and subsequently characterised to look at the virulence and infectivity of these strains compared to the wild type. In addition, several single mutations (*pspA, pspC, pspG*) and a double mutation (*pspA-E, pspG*) were constructed.

In *E. coli*, *psp* transcription is σ^{54} and *psp*F dependent and expression is controlled by a negative regulator (*pspA*) and several positive regulators (*pspC* and *pspF*) (3, 37, 261), however it is not known whether the same model applies to *S.* Typhimurium.

To further clarify Psp transcription in *S.* Typhimurium, σ^{E} regulated gene expression was investigated using the lacZ reporter gene to construct lacZ fusion promoters.

4.2 Mutagenesis of phage shock protein genes

To examine the role of the phage shock proteins in *S.* Typhimurium and their subsequent effect on virulence, several psp deletion mutants were constructed using Red mutagenesis to replace the gene of interest with a kanamycin resistance cassette as shown in method 2.7.2.

Firstly, a psp null mutant was made (SL1344 *psp*) replacing the entire psp operon (*pspABCDE*) with a kanamycin resistance cassette using oligonucleotides pspREDF and pspREDR to allow antibiotic selection and further to help identify our SL1344 *psp* strain during future *in vivo* experiments. PCR verification as shown in Figure 4.3 confirmed the correct mutagenesis of the psp operon. Single *psp* deletion mutations were constructed as above exchanging *pspA, pspC* and *pspG* with the kanamycin cassette using oligonucleotides pspAredF, pspAredR, PSPCREDF, PSPCREDR, pspGREDFor and pspGREDRev to produce SL1344 *pspA*, SL1344 *pspC* and SL1344 *pspG* respectively. PCR verification was performed as in 2.7.2, using oligonucleotides pspAEXTFor, pspAextRev, pspCextFor2, pspCextR, pspGextFor2, and pspGextRev2 (data not shown).

To construct a double mutation lacking the phage shock genes *pspABCDE* and also *pspG*, the original SL1344 *psp* was taken and Red mutagenesis performed allowing the exchange of the *pspG* gene for a chloramphenicol cassette. In this way, the SL1344 *psp pspG* double mutant could be differentiated from the original SL1344 *psp*. To ensure efficient mutagenesis of *pspABCDE* and *pspG*, PCR verification was performed as before using external oligonucleotides mentioned above.

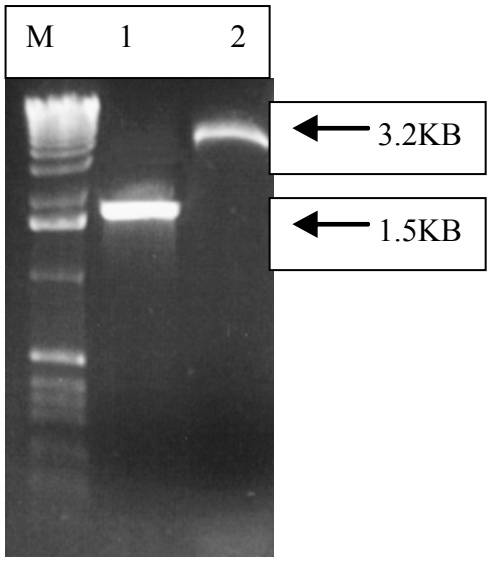


Figure 4.3 - PCR verification of an *S.* Typhimurium SL1344 psp mutant
Boilate PCR was performed on SL1344 *psp* (1) and SL1344 WT (2). PCR verification was performed as shown in 2.7.2, using oligonucleotides pspextF2 and pspextR2. All products were run on a 1% agarose gel and sized against a Hyperladder I marker (M). The SL1344 *psp* amplicon of 1.5Kb corresponds to the 1.5Kb kanamycin cassette inserted, and the SL1344 WT amplicon of 3.2Kb corresponds to the expected size of wild type *psp*.

4.3 *In-vitro* analysis of phage shock protein mutants *psp, pspA* and *pspC*

Following construction of SL1344 *psp,* and *pspC*, several phenotypic assays were set-up to examine the behaviour of these psp mutants compared to SL1344 wild type and to SL1344 *rpoE*. As extreme stress conditions are thought to be potential inducers of the psp system, growth curve assays were set up as described in 2.8.1 to examine growth at 48^{o}C, and also growth in salt, sugar and alcohol compounds known to affect the RpoE response of SL1344. Table 9 shows the results achieved from these assays, and it can be seen that under all conditions tested, SL1344 *rpoE* exhibited greatly reduced growth. In contrast, SL1344 *psp* and *pspC* strains showed no difference compared to SL1344 WT in the presence of sodium chloride, sucrose or 3% ethanol, however there was a difference observed in the growth at 48^{o}C, and in the presence of 5% ethanol.

Table 9. Growth of WT compared to *psp, pspC* and *rpoE* under extreme stress conditions.
This shows the comparable growth rates between SL1344 wild type, *psp*, *pspC* and *rpoE*. 0 indicates no difference in growth, ++ indicates a difference of between 20% and 40%, and +++ indicates a difference of 40% or greater.

Treatment	SL1344 WT compared to *psp*	SL1344 WT compared to *pspC*	SL1344 WT compared to *rpoE*
48° Growth	++	++	+++
0.6M NaCl	0	0	+++
0.6M sucrose	0	0	+++
3% ethanol	0	0	+++
5% ethanol	++	++	+++

These initial experiments were only carried out on a single occasion and as such no statistical information could be retrieved from this data.

Following examination of these results, a SL1344 *pspA* mutant was constructed and it was decided that growth at 48^{o}C appeared to be the best conditions to observe any phenotypic differences between SL1344 WT and the phage shock protein genes.

Figure 4.4 shows the growth curve achieved for each of these strains at 48^{o}C. Results show data obtained from one experiment. It should be noted at this point that these studies were performed before the arrival of the Bioscreen C machine and as such one replicate was set up on each occasion. Each growth curve experiment was repeated on three separate occasions however, and the same trend was seen on each occasion. From the data shown on Figure 4.4, it can be seen that all *psp* gene deletions are affected during growth at 48^{o}C to varying degrees compared to SL1344 WT. *rpoE* shows the most significantly affected growth defects followed by *psp*. This could be expected as this mutant lacks the entire psp operon, and not just single gene mutations in the case of *pspA* and *pspC*. *pspA* exhibits a slight reduction in growth in liquid media at 48^{o}C compared to SL1344 WT, while *pspC* exhibits a greater reduction in growth, though not as much as is seen in the entire psp null mutation.

It is known that *S.* Typhimurium *rpoE* shows increased sensitivity to H_2O_2 and paraquat (167) compared to WT. Analysis of sensitivity of SL1344 *psp* to 3% H_2O_2, 2% paraquat and 10% SDS compared to SL1344 WT was performed by disc diffusion assays as described in 2.8.2. The assays were performed at 30°C and 37°C as it has been shown that *rpoE* shows greater sensitivity to H_2O_2 at 37°C than at 30°C, and both *rpoE* and WT show greater sensitivity to paraquat at 30°C than at 37°C(167). The rationale behind these results is that temperature may affect the ability of bacteria to adapt to oxidative stress. There was no significant difference in sensitivity to H_2O_2

or 10% SDS between *psp* and WT. Figure 4.5 shows the results obtained from disc diffusion assays looking at inhibition of growth in the presence of 2 % paraquat at 30°C and 37°C. An ANOVA test using the graph pad statistical software was used to calculate levels of significant difference.

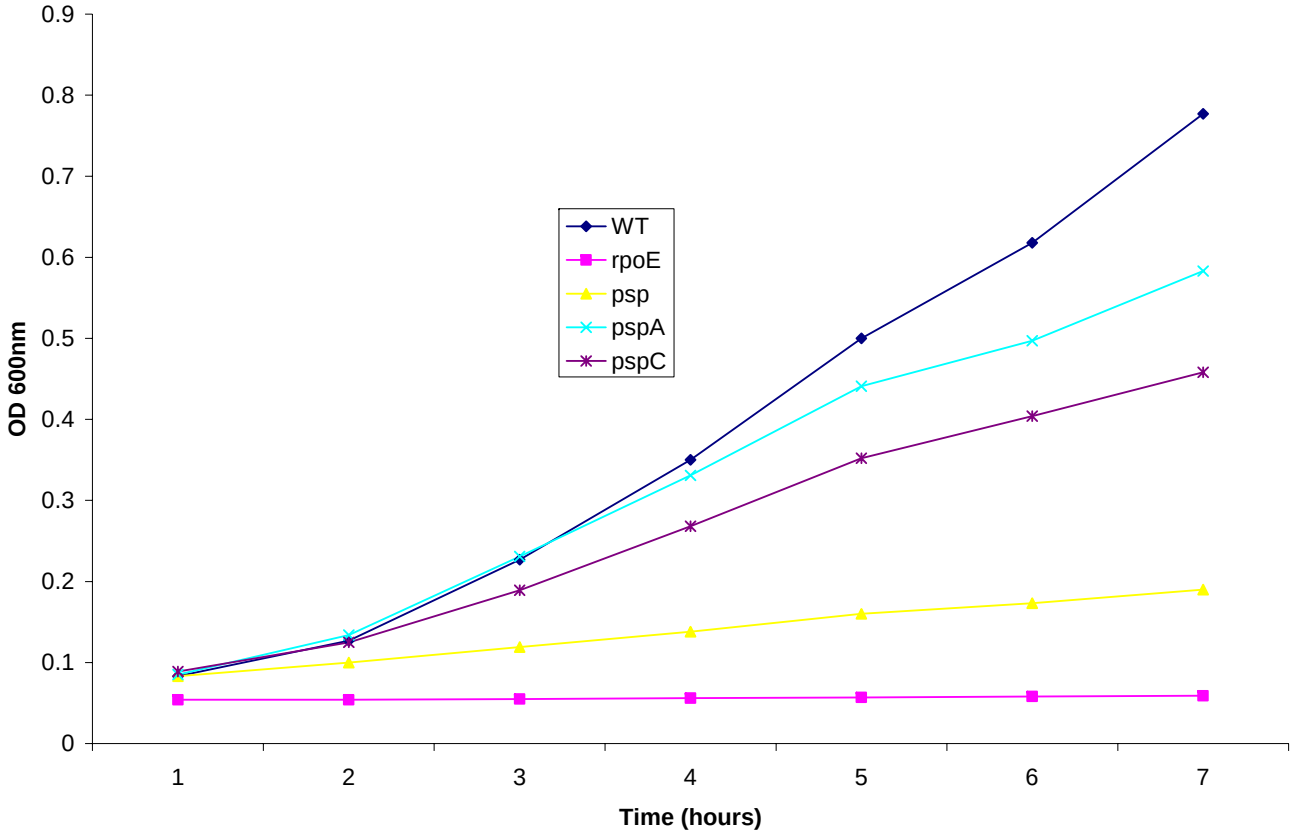


Figure 4.4 - Effect of *psp* mutations on the growth of *S.* Typhimurium in liquid media at 48°C
SL1344 *psp, pspA,* and *pspC* were compared with SL1344 WT and *rpoE* for their ability to grow in culture at 48ºC. Growth was monitored for 7 hours and measured spectrophotometrically at OD600nm. N=1.

Growth at 30 ^{o}C in the presence of paraquat is significantly different in *rpoE* and *psp* compared to SL1344 WT with both *rpoE* and *psp*.

Following on from the disc diffusion assays shown above, similar experiments were conducted to look at the relative sensitivies of our *psp* deletions using various antibiotics covering various modes of action (listed in table 7). There was no observed difference in the sensitivities of any of our *psp* deletions compared to a WT *S.* Typhimurium strain in response to all the antibiotics tested.

The sensitivity of our *psp, pspA,* and *pspC* deletions were then tested against the membrane damaging agent sodium cholate and also the protonophore CCCP (Figure 4.6 and 4.7) however again there was no significant difference seen between the sensitivities of any of our strains being tested.

4.4 Invasion and persistence of Psp mutants in a macrophage like cell-line

Previous studies with *S.* Typhimurium showed that an *rpoE* mutant strain did not survive as well in macrophages as SL1344 wild type (167). Also, the *psp* genes were identified in a microarray looking at the regulation of *S.* Typhimurium genes in macrophages (95). To investigate a possible role of *psp* in intracellular survival within macrophages and nonphagocytic cells, SL1344 *psp* was assayed for its ability of invade and survive in the murine macrophage cell line RAW264.7 and also the non phagocytic Hep-2 cells. Results showed no significant difference in the number of *psp* bacteria inside RAW264.7 cells compared to SL1344 WT. Similarly *psp* bacteria were also shown to invade Hep-2 cells normally (data not shown).

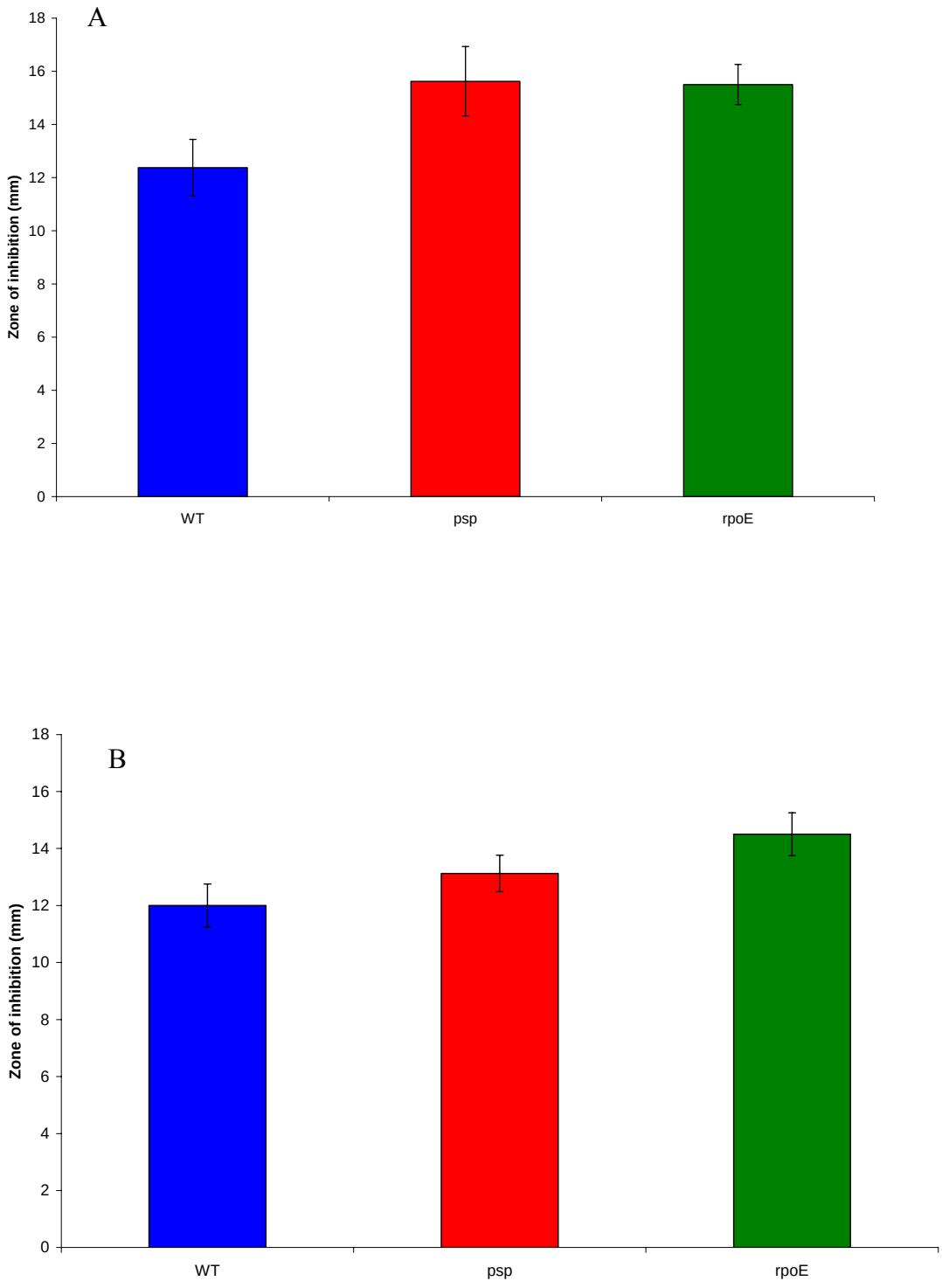


Figure 4.5 – Sensitivity of *S.* Typhimurium *psp* to paraquat
SL1344 WT, *psp* and *rpoE* strains were tested for their sensitivity to2 % paraquat by a disc diffusion assay. Bars represent the mean diameters of the zone of inhibition (mm) of 8 replicates whilst the error bars represent the standard deviation of the mean. (A) shows growth at 30^{o}C(B) shows growth at 37^{o}C.

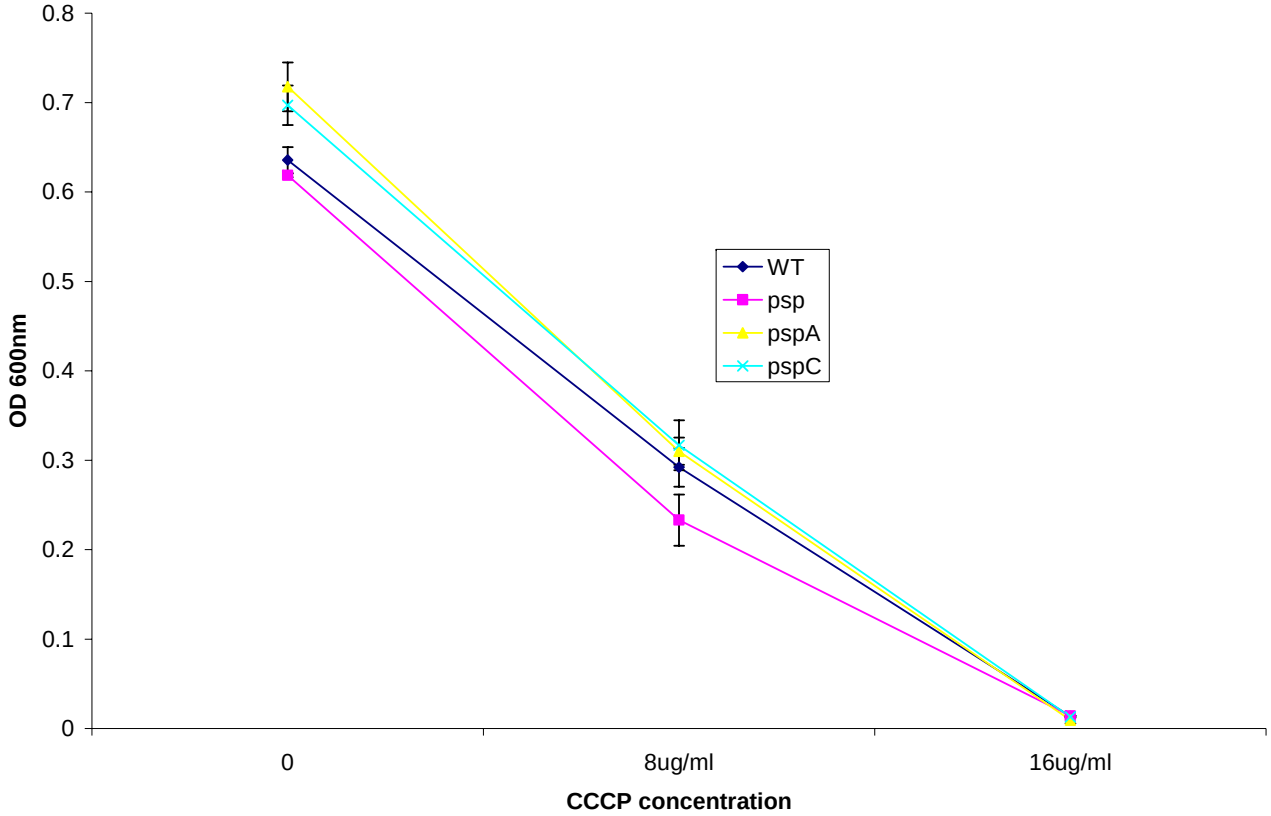


Figure 4.6 - Effect of *psp, pspA,* and *pspC* mutations on the growth of *S.* Typhimurium in liquid media supplemented with CCCP
The Psp mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 8, and 16µg/ml CCCP at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

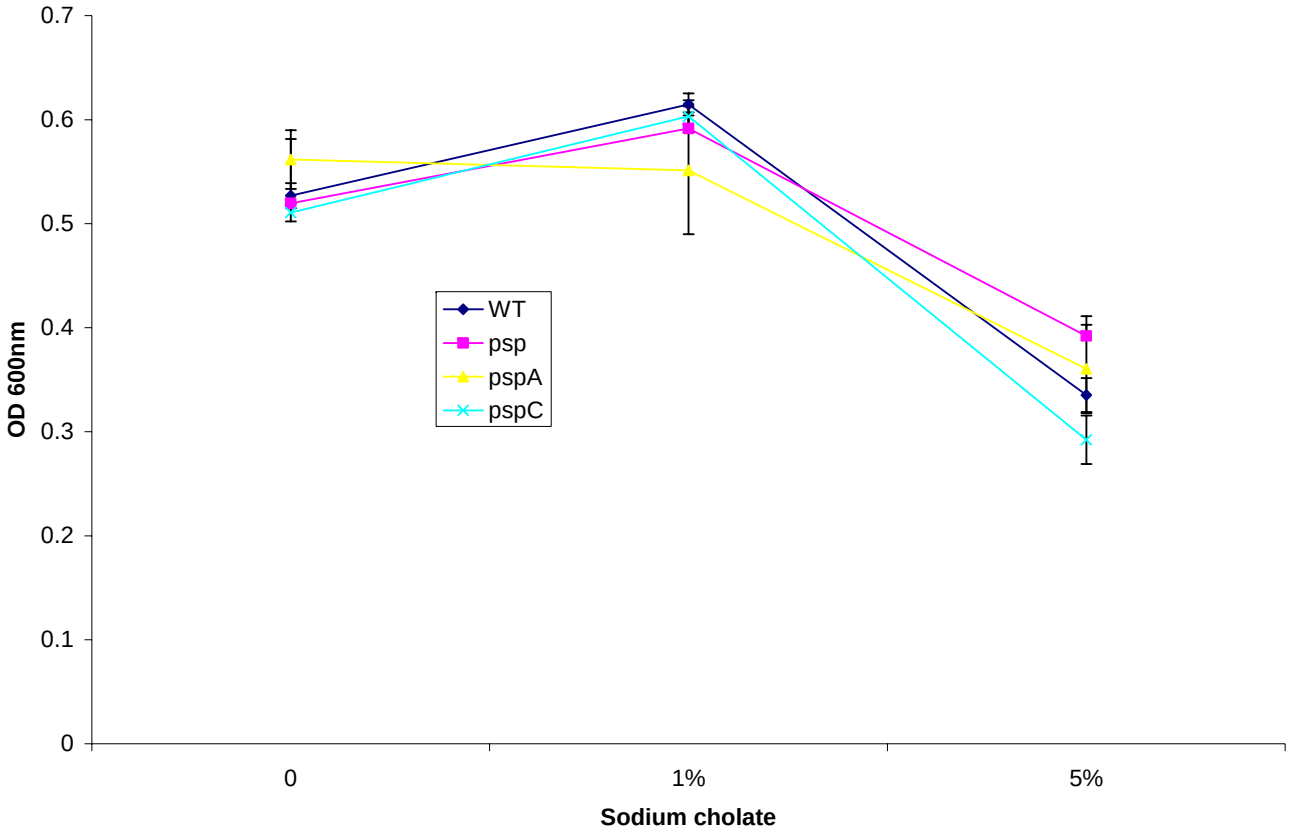


Figure 4.7 - Effect of *psp, pspA,* and *pspC* mutations on the growth of *S.* Typhimurium in liquid media supplemented with sodium cholate.
psp, pspA, and *pspC* mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 1, and 5% sodium cholate at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

4.5 *In vivo* analysis of *psp, pspA* and *pspC*

psp, pspA and *pspC* deletion mutants were investigated to study any role of virulence *in vivo* from competition assays as detailed in 2.8.5.1. Briefly, mice were given approximately 10^3 CFU of both wild type *S.* Typhimurium and the mutant strain under investigation by IP injection. Organs were removed and bacterial counts performed on day three. A CI of ~1.0 indicates that the strains compete equally well and the lower the CI value the more attenuated the strain under study. Figure 4.8 shows the competition assay results obtained for *psp, pspA* and *pspC*. As these results show, only *psp* is significantly attenuated, with a CI of 0.37 and 0.4 respectively for CFU counts from spleen and liver. A paired T- test was performed on the bacterial counts obtained from each organ to observe if the mean of the differences between SL1344 *psp* and WT differ significantly from zero. The two-tailed P value is 0.0001, which is considered extremely significant. As only the entire SL1344 *psp* mutant showed significant levels of attenuation in the IP model of infection, this strain was chosen for further *in vivo* study.

Oral challenge assays were established as shown in 2.8.5.2 with two groups of mice inoculated with either SL1344 *psp* or WT, via an oral infection route at a concentration of 5 x 10^7, or 5 x 10^5 per ml respectively. The number of CFU obtained from various organs per group was determined on day 3 and 6 post infection. Results from this assay (shown in Figure 4.9) show slight attenuation of the *psp* mutant compared to the WT strain on day 6 post infection.

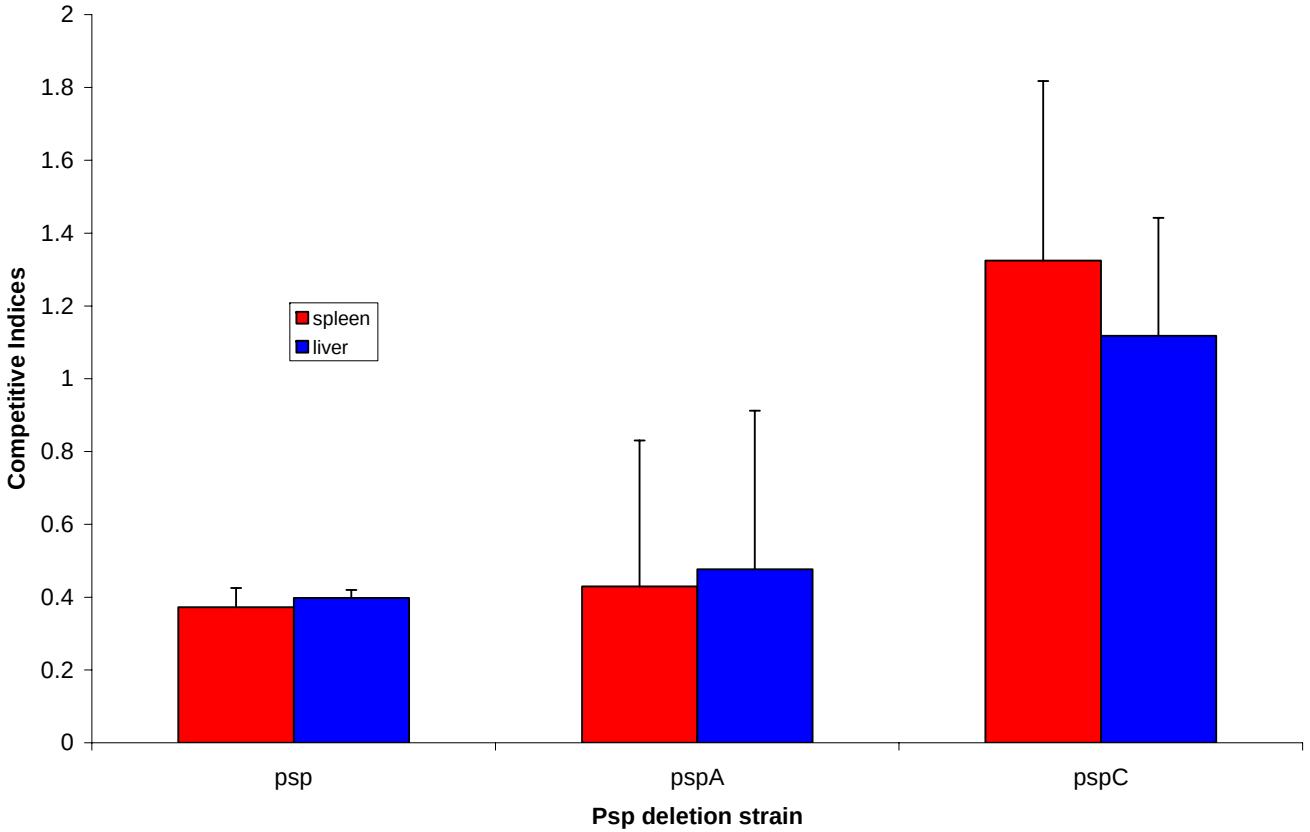


Figure 4.8 - Competition assay of SL1344 WT and psp, pspA and pspC
Groups of 5 mice were challenged with a mixed dose of ~10^3 CFU SL1344 WT and the appropriate mutant strain via IP injection. Data is displayed as the average competitive indices. Bacterial counts were performed on day three. Error bars represent the standard deviation of the mean.

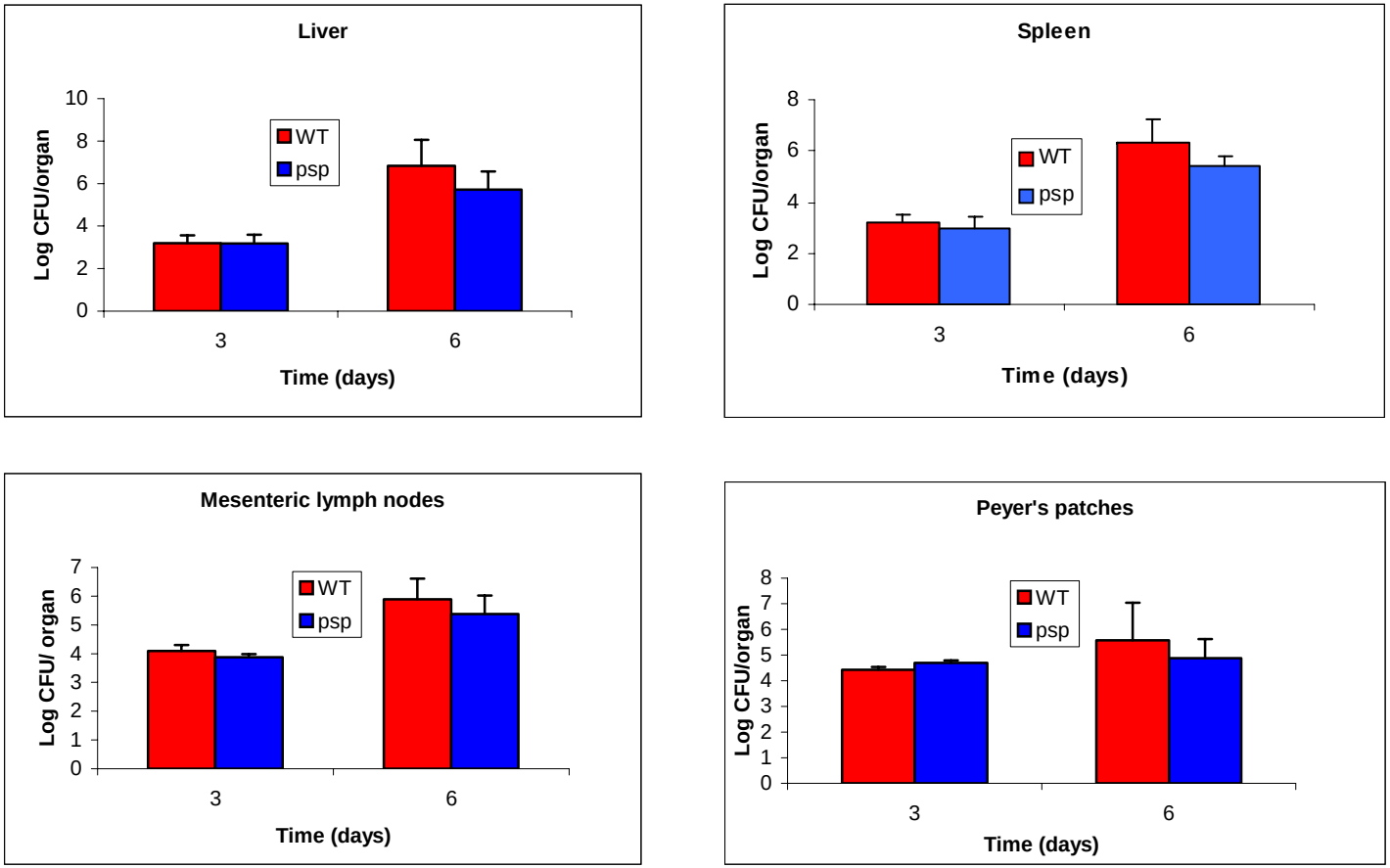


Figure 4.9 - Oral Infection of BALB/c mice with wild type *S.* Typhimurium, and a *psp* mutant

Single doses of either $5x10^5$ CFU or 5×10^7 CFU per ml were administered by oral gavage and CFU/organ counts performed on 3 and 6 days post infection. Data presented is a mean of 5 mice and error bars represent the standard deviation of the mean.

4.6 *In vitro* analysis of *pspG*

During my study, and following construction and characterisation of *psp, pspA* and *pspC* deletion mutations, several papers highlighted the recent discovery of *pspG* (131, 222). *Y. enterocolitica pspG* was identified as a new member of the *pspF* regulon and, similarly to *pspA*, it is positively regulated by *pspF*, negatively regulated by *pspA* and induced in response to the production of secretins. A *pspG* null mutation was also found to exacerbate the phenotypes previously associated with loss of *pspA* in *Y. enterocolitica*, such as a defect in growth following production of the secretin *yscC* and attenuation in a mouse model of infection (131).

As such, I constructed a SL1344 *pspG* single mutant and a SL1344 *psp pspG* double mutant as described in 2.7. An honours year student working in our laboratory was involved with the characterisation of these mutants.

Five SL1344 *S.* Typhimurium strains, *psp, pspC, pspG, psp pspG* and WT, were grown to mid-exponential phase under SPI-1 conditions (shown in section 2.2) to establish whether the psp response has a role in exponential growth. Under these growth conditions, no detrimental effects on growth were observed for any of the mutant strains, indicating that the *psp* genes do not have a role in *S.* Typhimurium growth during the exponential phase.

4.7 Construction and activity of psp promoter reporter plasmids.

To study regulation of both *pspF* and *pspABCDE*, promoter constructs were designed using the 200bp section of intergenic DNA located between *pspF* and *pspA*. This region was chosen as it is directly upstream of *pspA* and the likely location of the promoter and it was cloned in the forward and reverse direction to investigate both pspF and pspABCDE expression. Promoter constructs were created using standard

molecular cloning techniques as described in section 2.4. Cloned regions of DNA covering the intergenic region between *pspA* and *pspF* were PCR amplified using oligonucleotides pspAFF, pspAFR, pspFAF and pspFAR respectively and fused to a promoterless copy of *lacZ* in plasmid pTL61t on HindIII and XbaI restriction enzymes sites incorporated into the oligonucleotides. The construct covering the expected pspF promoter was called p.pspF, while the construct covering the pspA promoter was called p.pspA1. These constructs were transduced into SL1344 WT, *rpoE* and *rpoN Salmonella* strains. The presence of insert was verified by size discrimination of PCR products obtained as illustrated in Figure 4.10. A product of size 300bp is seen in the empty vector control, while a 500bp product is seen for both constructs containing the 200bp promoter construct. Following construction of these two lacZ promoter constructs, β-Galactosidase assays were performed as described in section 2.5.

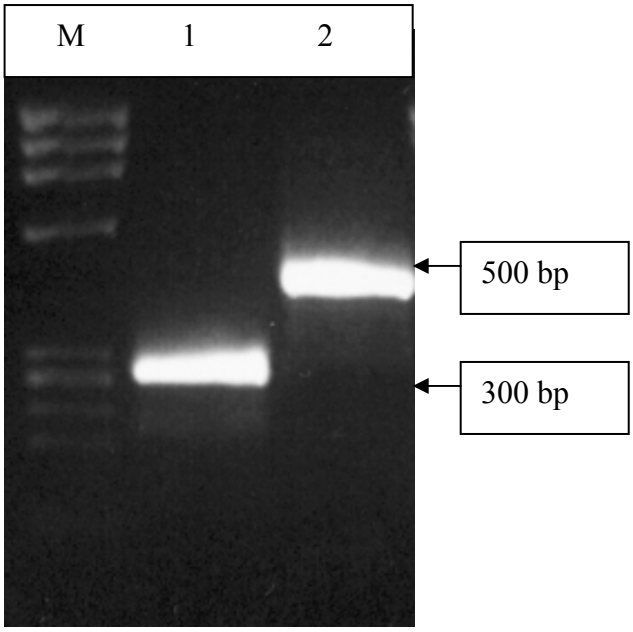


Figure 4.10 - PCR Verification of p.pspF insert in pTL61t vector.
Lane M is the 100bp molecular weight marker, lane 1 shows the amplification of the pTL61t empty vector, and lane 2 shows the amplification of p.pspF insert in pTL61t vector. All products were run on a 2% agarose gel.

Results from these studies (Figure 4.11) indicated expression of *pspF* is under negative regulation by *rpoN*. At the later time point, there appears to be more activity in our *pspF* construct when in WT compared to the *rpoE* mutant indicating that *rpoE* may be acting as a positive regulator in stationary phase, though further verification is required.

Initial studies showed no β-Galactosidase activity was observed in cells containing the p.pspA1 construct (responsible for expression of *pspABCDE*). It was thought that this could perhaps be due to the pspA promoter being further downstream of pspA than I had originally hypothesised and as such a longer region of DNA extending 100bp further downstream of the intergenic region was amplified and cloned as before using oligonucleotides pspFAFlong and pspFARlong. This longer construct was named p.pspA2.

β- Galactosidase assays using the p.pspA2 construct however also proved inconclusive, yielding β-galactosidase activity to a similar level as was obtained in an empty vector pTL61t. As there appeared to be no difference in the level of activity in our small and long pspA constructs, for further studies I reverted to using the original short construct p.pspA1. At this stage, as all studies using our p.pspA1 construct were negative, I adapted the β-Galactosidase assay to mimic the conditions used in our original microarray which identified *pspABCDE* genes as being up-regulated in an over-expressing RpoE strain (RpoE+) compared to SL1344 WT during log phase. For this assay I transduced p.pspA1 into SL1344 WT, *psp* and *pspA* and examined the β-Galactosidase activity at both log phase and stationary phase and in the presence and absence of the *rpoE* inducer arabinose.

Results from this assay (shown in Figure 4.12) showed an increase in β-Galactosidase activity of p.pspA1 in SL1344 WT RpoE+ induced with arabinose compared to the

same strain in the absence of arabinose at both log phase and stationary phase. As such, this would confirm what was seen in the original microarray data where pspABCDE expression is up-regulated in WT SL1344 RpoE+ induced with arabinose. There was no difference observed in the activity levels between empty vector and p.pspA1 *psp* RpoE+ in either the presence or absence of arabinose, as expected due to the deficiency of phage shock protein genes.

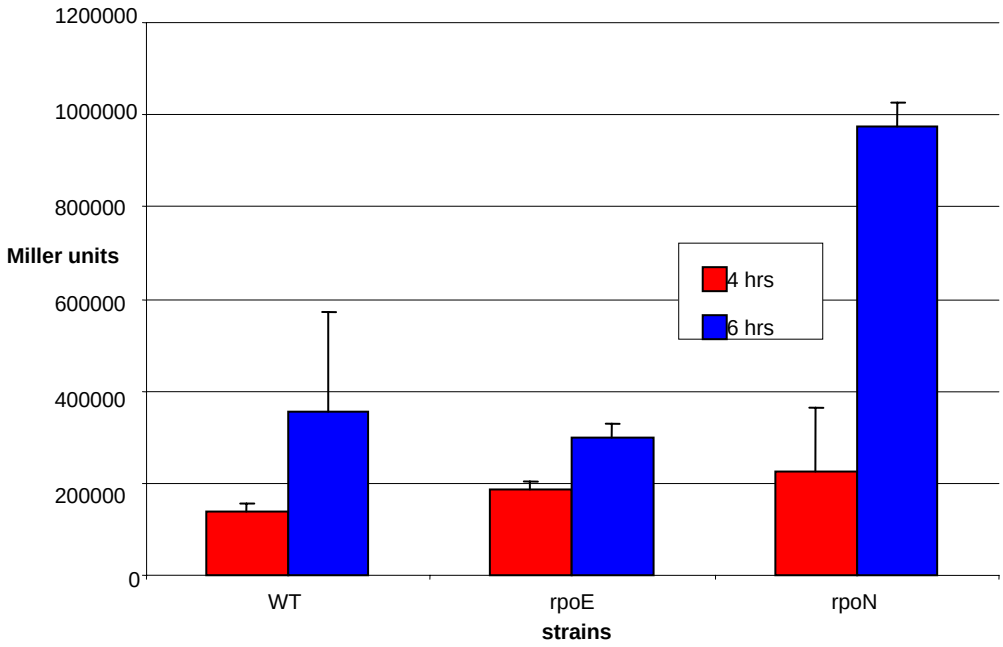


Figure 4.11 – β- Galactosidsase activity of PspF
Expression of PspF was assayed by β-Galactosidase activity measured in strains carrying a p.pspF-lacZ transcription fusion. Promoter activity of pspF was assayed during mid exponential and stationary phase in WT, *rpoE*, and *rpoN*. Experiments were performed in triplicate and the error bar indicates the standard deviation.

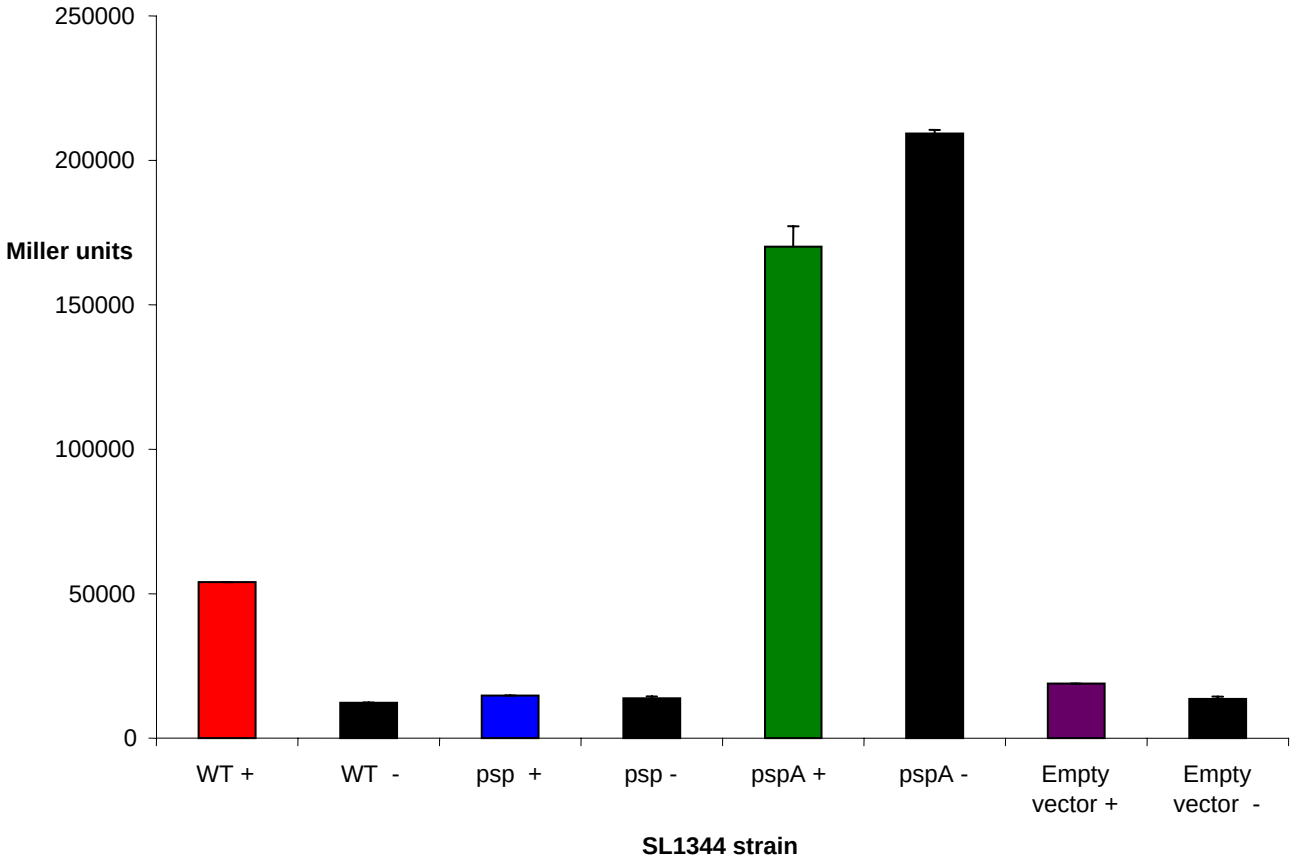


Figure 4.12 - β- Galactosidsase activity of PspA in various *Salmonella* strains
Expression of PspABCDE as given by β-Galactosidase activity measured in strains carrying a p.pspA1-lacZ transcription fusion (in WT, *psp*, and *pspA*) and empty vector. All strains contained the over-expressing RpoE arabinose inducible promoter. Activity was measured in mid-log phase LB cultures with the addition (+) or absence (-) (diagonal lines) of 0.2% arabinose in certain samples.
Experiments were performed in triplicate and the error bar indicates the standard deviation.

There was a huge difference observed in the activity of p.pspA1 *pspA* RpoE+ compared to p.pspA1 WT RpoE+ in both the presence and absence of arabinose, indicating that this increased expression of pspABCDE in a *pspA* strain compared to WT is independent of RpoE.

As there has been recent research showing that overexpression of *yggT* (an integral membrane protein) can induce psp in *Yersisinia enterocolitica* (236) the above experiment was repeated in a SL1344 *yggT* null strain, however this proved inconclusive as in both the presence and absence of arabinose, there was no difference in activity levels compared to empty vector.

4.8 Quantitative Real-Time PCR

RT-PCR was performed as described in 2.11 for further study of PspABCDE expression. RNA was made from SL1344 WT, *rpoE* and SL1344 WT containing RpoE+ as described in 2.9. Oligonucleotides pspARTfor and pspARTrev were designed for amplification of *pspA*. Results from this experiment showed an approximate two fold difference in pspA expression in SL1344 WT RpoE+ compared to SL1344 WT.

4.9 Discussion

The phage shock protein (PSP) response in *S.* Typhimurium was chosen for further investigation as the *psp* genes were highly up-regulated in microarrays when RpoE was over-expressed or when *S.* Typhimurium was intracellular within macrophages (95). We hypothesised from our array and the macrophage array data that the PSP response may play an important role in the virulence of *S.* Typhimurium and as such I constructed several null mutations of the *psp* operon for characterisation (SL1344 *pspA-E*, *pspA, pspC pspG* and *psp pspG*).

Much of the recent research performed on the PSP response has concentrated on *E. coli* or *Y. enterocolitica* and initial phenotypic assays were based on inducers known to affect the PSP response in either of these bacteria. The PSP was first described in 1990 (38) and in this study environmental inducers of PSP production were found to include extreme heat-shock, high concentrations of ethanol, and hyperosmotic shock. These environmental conditions were chosen in the first set of phenotypic assays undertaken when characterising the SL1344 *pspA-E* and *pspC* mutants, the first *psp* deletion mutants I constructed. Results from these studies showed that a *S.* Typhimurium *rpoE* null mutant is negatively affected by all of the environmental conditions tested and both the *pspA-E* null and *pspC S.* Typhimurium deletion strains are negatively affected by high temperatures and high ethanol concentrations. Experiments studying growth at 48ºC were only performed on a single occasion and require repeating to ensure that this temperature sensitive phenotype observed in all our *psp* mutant strains is accurate. Growth of the *pspA* deletion strain is negatively affected at 48ºC, though not to the same level as the *pspA-E* deletion mutant(Figure 4.4) which correlates with the findings in *E. coli* (38). No difference was seen in any

of the *psp* deletion strains compared to the WT strain when under osmotic shock, contrary to what is seen in *E. coli*. To investigate this further it would be useful to test our strains under varying osmolarity.

I investigated the effect of CCCP, hydrogen peroxide, sodium cholate and SDS on the *S.* Typhimurium *psp* mutant and no difference was observed between the SL1344 *pspA-E*, *pspA*, *pspC* and WT strains. Increased sensitivity of *S.* Typhimurium *rpoE* and *pspA-E* mutants to 2% paraquat compared to WT was seen and for both mutants a greater sensitivity was observed at 30ºC compared to 37ºC which correlates with past research within our laboratory (167) showing an *rpoE* mutant strain to be more sensitive to paraquat at 30ºC than 37ºC. It is thought that this difference may be because at higher temperatures there is increased expression of intra- and extracytoplasmic stress regulons providing greater resistance to oxidants. The effect of 2% paraquat on our *psp* mutant appears to be the same as is seen in our *rpoE* mutant.

The sensitivity of *pspA-E*, *pspA* and *pspC* mutants to the various antibiotics listed in table 6 was tested. There was no difference in the sensitivities of any of the *psp* mutants and the WT strain, this is in agreement with past research using a limited range of antibiotics affecting DNA which found an *E. coli psp* mutant to be unaffected by novobiocin, nalidixic acid and mitomycin C. It is coming to light that the PSP response is a separate ESR in itself and I hypothesise that this response may be affected by signals that may not elicit a response in the RpoE ESR and vice versa. It would be useful to investigate the response of our *psp* mutant strains to a greater number of compounds and another area that could be developed further would be to investigate the LPS profiles of our *psp* mutant strains to investigate if the *psp* genes have a role in LPS biogenesis.

In summation, *S.* Typhimurium strains containing a mutation that deletes the entire *psp* operon or single *psp* genes (*pspA* or *pspC*) are primarily affected under extreme stress conditions such as high temperature and high concentrations of ethanol. The greatest difference is observed in the *pspA-E* null mutant which lacks the entire *pspA-E* operon, compared to either the *pspA* or *pspC* single mutants, though these single mutations do appear to have an effect to a smaller degree. A recent study of PSP inducers in *Y. enterocolitica* (236) found PSP to be induced distinctly from the RpoE system and it is likely that the *psp* genes constitute a fourth Extracytoplasmic Stress Response (ESR), rather than a set of genes regulated by the RpoE extracytoplasmic stress response.

The *psp* genes in *S.* Typhimurium are up-regulated in macrophages (95) and a *S.* Typhimurium *rpoE* mutant strain is known to have a reduced ability to invade and survive within macrophages (167). Therefore I investigated a possible role of the PSP response in intracellular survival within macrophages and nonphagocytic cells. SL1344 *pspA-E* was assayed for its ability to invade and survive in the murine macrophage cell line RAW264.7 and also the non-phagocytic Hep-2 cells. Results showed no significant difference in the number of SL1344 *psp* bacteria inside RAW264.7 cells compared to SL1344 WT. Similarly, bacteria were also shown to invade Hep-2 cells normally (data not shown). The RpoE response in *Salmonella* is thought to have a role in the primary stages of infection of macrophages, however if the PSP response is a separate ESR it is conceivable that the PSP response in *Salmonella* may affect macrophage infection at a different stage to that observed for RpoE and future studies could examine if the *psp* mutations affect *S.* Typhimurium infection of eukaryotic cells at later times.

To investigate whether the PSP response in *S.* Typhimurium plays in role in virulence infection studies were performed. Mutations in *Y. enterocolitica psp* genes has an effect on the virulence in mice in a competition assay, with a single *pspC* mutation appearing to cause a greater effect than an entire *psp* null mutation (69). It is known that the *psp* locus of *Y. enterocolitica* is required for virulence when the Ysc TTSS is produced with *psp* gene expression being induced when YscC (a secretin protein of the Ysc TTSS) was expressed (69). *S.* Typhimurium possesses 2 TTSS and it would be interesting to over express for example InvG, a secretin protein of the SPI-1 TTSS and look at its effect on the expression of *psp* genes and the growth of *S.* Typhimurium *psp* mutants.

In vivo competition assays were performed on SL1344 *pspA-E, pspA* and *pspC* with only the SL1344 *pspA-E* strain appearing to be attenuated (Figure 4.8). This is in contrast to what is seen in *Y. enterocolitica* (69) however it is likely that these two different bacteria operate in very different ways at the molecular level. It should be noted however that there is a large amount of variation in the C.I (competitive indices) in the *pspA* and *pspC* groups which may be due to the small sample number of mice. The reason for this is unknown but repeating the experiment with larger groups of mice should reduce the variation. Of interest is the fact that this is seen in both the liver and spleen and not just a single organ. To gain more confidence in these results it would be useful to repeat these experiments using a larger number of mice. This large degree of variability is not seen in the *pspA-E* strain so it would be interesting to discover if on a repeat experiment, *pspA* and *pspC* again show high variability.

To investigate further the effect of the PSP response on virulence, single infection studies were performed showing SL1344 *pspA-E* to be attenuated compared to WT at six days post infection. This attenuation is not observed earlier in the study (at day 3)

and as such I hypothesised that PSP is important to *S.* Typhimurium at later stages of infection. This could explain why no difference was seen in our macrophage assay studies where infection is followed up to a maximum of 24 hours. The nature of these single studies observing oral infection require mice to be infected with a 100 fold log lower dose of WT compared to our strain under study therefore although our data is dose corrected it is possible that this differing dose may be the reason for the attenuation being seen on day 6 not being observed on day 3. I suggest that the attenuation observed at day 6 indicates that the PSP response is necessary for the ultimate virulence of *S.* Typhimurium and is more likely to be of importance at the replication and persistence stages on infection than in invasion and attachment of the bacteria. This is contrary to what is seen in the RpoE response and indicates further that the PSP response is an ESR independent of the RpoE ESR. Characterisation of the numerous *S.* Typhimurium *psp* mutants leads me to believe the PSP response is a fourth ESR induced upon high stress conditions which plays an important virulence role in the sustainment of *Salmonella* infection. To investigate this in more detail given more time I would try and characterise the phenotypes of our *psp* mutants in more detail, using a greater variety of compounds. In particular I would study the LPS profile of each of our strains to see if absence of PSP affects LPS expression or O-antigen side chain length. I would also examine if absence of PSP affects secretion of SPI-1 and SPI-2 effectors and perform studies on the outer membrane protein profiles. It would be interesting to combine *psp* mutations in both an *rpoE* mutant and also in an over expressing RpoE strain and observe the outer membrane protein variation between strains. *pspG* was discovered to be a member of the *psp* operon towards the end of my studies and as such there is a lot more characterisation still to be done on this gene including its interactions with other members of the *psp* operon.

Final characterisation of the PSP response in S. Typhimurium involved analysis of *psp* gene expression. There has been much study on the regulation of the *psp* operon in *E. coli* (37, 38, 67) however it is likely that differences exist between the PSP system in *E. coli* and that of different enteric bacteria. For example it is known that the *rpoE* gene is essential in both *E. coli* (74) and *Y. enterocolitica* (158), yet not in *S.* Typhimurium (167). Also, it is known that in *Y. enterocolitica* a single *pspC* mutation shows greater attenuation in a mouse model than an entire *psp* null mutation (68) however my studies have shown the opposite to be true in *S.* Typhimurium. The *psp* operon contains genes that can positively and negatively regulate its own expression, which together with data showing that inactivation of *pspC* in *Y. enterocolitica* is more attenuating than the loss of the entire *psp* operon highlights the complexity of the interactions between PSP members.

It has been established that in *E. coli pspA–E* transcription is driven by an *rpoN* dependent promoter upstream of *pspA* (39, 261). PspA is a negative regulator of the PSP system (80, 87) and PspB and C co-operate together as positive regulators of PSP (3). PspF is expressed independently of *rpoN* by a σ^{70} promoter which overlaps the *pspF* enhancer binding sequences (EHB) UASI and UASII (91, 184, 185, 392). It has recently been found that 6S RNA can directly inhibit *pspF* transcription leading to inhibition of *pspABCDE* and *pspG* expression (370). An interesting point to note is that in *E. coli* RpoE is not required for *psp* operon induction (261). In addition to our original microarray data, microarray analysis of stationary-phase gene expression in a *S.* Typhimurium *rpoE* mutant showed there was a large increase in the expression of *pspA* compared to WT (17) so it would appear that the PSP response is induced when the RpoE regulon is activated but also by the loss of RpoE activation. A 74-fold

increase compared to WT was seen in the above microarray experiment of stationary-phase gene expression in *pspA* in a *S*. Typhimurium *rpoE* mutant while our own studies showed an approximate 4 fold increase in *pspA* expression in a S. Typimurium over-expressing RpoE strain compared to WT.

To investigate the expression of *pspF* and *pspA-E*, *lacZ* promoter fusions were constructed by fusing the intergenic region between *pspA* and *pspF* to a promoterless copy of *lacZ.* My results indicate that expression of *pspF* is under negative regulation by *rpoN* (Figure 4.13). A slight increase in the activity of *pspF* in SL1344 WT compared to SL1344 *rpoE* at the second time point indicates that RpoE may be acting as a positive regulator of *pspF* in stationary phase, though further verification is required as there is a high level of variability in the data from our WT sample making the data difficult to interpret with confidence. This is in contrast to what is seen in *E. coli*, where *pspF* transcription is driven by an *rpoD* promoter, and is independent of RpoN. Further studies on the role of RpoE in regulation of *pspF* expression are warranted. We could not identify potential RpoE-dependent promoter sequences in the *pspF-pspA* intergenic region. This suggests that if RpoE is regulating *psp* gene expression, it is doing so indirectly.

The expression of *pspA–E* proved more complicated with several attempts made at constructing a *lacZ* promoter fusion containing the intergenic region between *pspA* and *pspF*, thought to be driving the expression of *pspA–E*. Initial β-Galactosidase assays proved negative, following which I adapted these assays to mimic conditions used in the original microarray. This showed an increase in *pspA-E* expression in a *S*.Typhimurium WT RpoE + strain (containing our *lacZ* promoter fusion) induced with arabinose compared to the same strain in the absence of arabinose at both log

phase and stationary phase (Figure 4.14) which would confirm what was seen in the original microarray data and indicates that the *lacZ* promoter construct did contain the correct promoter region.

Similar studies were set up to investigate *pspA-E* expression in a *S.* Typhimurium *psp* null mutant. These studies found no β-galactosidase activity in the *psp* null rpoE+ mutant in either the presence or absence of arabinose. This *psp* null mutant contains both the over-expressing *rpoE* plasmid and the *lacZ* promoter fusion. It would be expected that no activity was observed in this strain as this null mutant lacks all the *psp* genes required for Psp expression.

There was much greater *pspA-E* expression seen in a *pspA* mutant RpoE+ strain compared to WT RpoE+ in both the presence and absence of arabinose indicating that this increased expression of *pspABCDE* in a *pspA* null strain compared to WT is independent of RpoE which corresponds with my thoughts that the PSP system is an ESR system itself which can be both regulated by RpoE and also regulated independently of RpoE. It could be that in a *pspA* null strain the lack of *pspA* causes extreme stress to the cell resulting in huge over expression of *psp* independent of the effects of arabinose. RT-PCR was subsequently carried out which confirmed that *pspA* expression is greater in the SL1344 WT RpoE+ strain compared to SL1344 WT. Recent data has shown an *rpoE* mutation to result in increased *pspA* expression (measured by both RT-PCR and β-galactosidase assays) (17) which indicates that the expression of pspA-E is more complex than first thought.

Looking at our PSP expression studies it appears that regulation of *pspA–E* in *Salmonella* is complicated however this is to be expected as it is now coming to light

that the *psp* operon encodes an independent extracytoplasmic response system that can be affected by conditions that induce other ESR systems such as the RpoE response, however it can also be induced upon conditions that are independent of *rpoE* or *rpoN*. To gain a more complete understanding of the regulation of the PSP system in *S.* Typhimurium much more work is required. Studies that would clarify this further would be to look for the presence of potential promoters up or downstream of the *psp* operon and also to examine the inter-play between each of the *psp* genes and their subsequent role in the regulation of the *psp* operon as a whole. Future studies could be established to construct putative *psp* promoter *lacZ* fusions. It would also be useful to map the transcription start site of *psp* and a generic promoter consensus sequence could be devised from sequence data of known promoters and used in a promoter consensus search. It is hoped that the use of quantitative RT-PCR could also yield more confirmative data on the expression of Psp in *Salmonella*.

Chapter Five – Characterisation of the Cpx system in *S.* Typhimurium

5.1 Introduction

The CpxAR two component signal transduction pathway is a second extracytoplasmic stress response system known to be present in *Salmonella, E. coli* and *Yersinia* species which is discussed in detail in chapter one. Interestingly, a study designed to investigate the distribution of *cpxA, cpxR* and *cpxP* (CpxP is a periplasmic protein that regulates the CpxAR system) in prokaryotic genomes, discovered many common bacterial species that did not have homologues of these genes (*Campylobacter*, *Staphylococci*, and *Vibrio* species to name a few) (76). The CpxAR response system was first described in 1980 with the identification of Cpx mutants (238), individual Cpx proteins were then described and through their amino acid sequence, their precise role was subsequently identified (238, 358). CpxA is now known to be the membrane sensor that can sense extracytoplasmic stress, while CpxR is the response regulator. The system acts like many two component signal transduction pathways with a sensor kinase and a cognate response regulator that work together to sense and respond to changes within the cell. Further work has shown that outer membrane proteins such as NlpE (a new outer-membrane lipoprotein) could activate the Cpx signal transduction pathway through the CpxA sensor (82, 259, 350) and the Cpx system is also known to be induced by the increased expression of pili components (169), alkaline pH (313), and the expression of a folding-defective MalE protein (170). It is also known that the Cpx system can be inhibited by the binding of CpxP (a periplasmic protein) to CpxA (64).

Initial research on the Cpx system in *E. coli* identified *htrA, dsbA* and *ppiA* as the first Cpx regulated genes (63, 65) and a more recent study in 2002 identified 8 new target operons following a genome-wide promoter search (78).

Studies within our laboratory moved an important step forward with the confirmation of the role of the two-component regulator CpxAR in the virulence of *S.* Typhimurium (165). This research proved to be the first demonstration that the Cpx system is important for the *in vivo* virulence of a bacterial pathogen.

As the CpxAR system is only present in a limited number of bacterial species, it would be of interest to try and investigate why this system may only be of value to certain bacterial species.

To characterise the CpxAR response system in *S.* Typhimurium more comprehensively, I designed a microarray experiment to identify CpxAR regulated genes.

For use in the microarray, RNA was isolated from four Cpx associated strains allowing two comparisons of Cpx activation vs. non-activation. A SL1344 CpxAΔ strain was compared against a SL1344 CpxA* (constitutively expressed) strain, and two SL1344 WT strains containing the inducible nlpE pND18 plasmid (either non-induced or induced with arabinose). This chapter describes the design, analysis and subsequent interpretation of the Cpx microarray experiment.

5.2 Cpx Microarray Design and analysis

A microarray was set up in collaboration with Maria Fookes at the Sanger centre to identify putative Cpx regulated genes.

RNA was isolated from strains SL1344 *cpxAΔ*, SL1344 CpxA* (constitutively expressed) and two SL1344 WT strains containing the inducible *nlpE* pND18 plasmid (either non-induced or induced (*) with arabinose) as described in 2.9. These strains were chosen to allow two separate comparisons of induction and non-induction of the Cpx system. CpxAΔ vs CpxA* behave as if there is no CpxAR activity vs the CpxAR activity being permanently switched on respectively. In addition to this, we chose to compare WT SL1344 with or without the induction of NlpE (an OMP lipoprotein known to activate the Cpx system). Our reasoning for choosing these two different routes of Cpx induction was because we felt using the CpxA* strain alone may not be ideal as this strain does not appear to grow well and its growth can lead to the development of two colony types which may have secondary mutations that affect our results. As such, we choose to also study induction and non-induction of the Cpx system via the NlpE lipoprotein.

Following RNA isolation, all samples were sent to Maria Fookes at the Sanger centre for reverse transcription, labelling and hybridisation to the Generation 3 *Salmonella* microarray slides as illustrated in 2.10.1

Briefly, the work carried out at the Sanger centre was as follows: RNA was fluorescently labelled with Cy3 or Cy5 dyes and dye-swap experiments were performed for all hybridizations. Labelled cDNA was purified, precipitated and the resulting probes hybridized to the *Salmonella* microarray slide overnight. After washing, hybridization results were analysed using a Genepix 4000B scanner and quantified using Genepix Pro software.

In the analysis of our microarray data we defined genes as being putatively up or down regulated being those genes that had a two fold ratio up or down and/or a t-test p-value of less than 0.05. We decided upon this selection system as it maximised the

number of putative genes we had to investigate further. It should be noted that compilation of genes by the more stringent criteria of selecting genes with both a two fold ratio up or down and a t-test p-value of less than 0.05 did not correctly identify genes known to be Cpx regulated in *E. coli* such as *htrA* due to their un-expected high p-value and as such this criteria was disregarded. The 1309 genes identified from our microarray Cpx experiment as being up or down regulated are shown in Appendix A.

5.3 Identification of Cpx Regulated Genes

For discussion in this chapter I devised 2 tables listing genes of interest which were putatively identified as either Cpx up-regulated (shown in table 10) or Cpx down-regulated (shown in table 11). All genes within these tables have a normalised ratio greater than approximately 2 or less than 0.5 in our Cpx microarray experiment for either WT vs WT induced or cpxA * vs CpxAΔ.

Table 10 – A selection of genes from Cpx Sanger array comparing WT vs WT induced (both contain plasmid and CpxA* vs CxpAΔ with a normalised ratio greater than approximately 2. Entire data from this microarray experiment can be found in appendix A of this thesis. Previously known members of the Cpx regulon are asterixed (*).

Gene name	Gene function	Systematic name	Fold Induction WT vs WT*	t-test P-value	Fold induction CpxA* vs CpxA	t-test P-value
chaA	Putative calcium/proton antiporter	STM1771	2.501	0.042	3.495	0.159
tgt	tRNA-guanine transglycosylase	STM0405	1.560	0.412	2.045	0.089
*htrA**	Heat shock protein htrA	STM0209	2.435	0.352	3.137	0.178
tolQ	TolQ protein	STM0745	1.955	0.086	1.805	0.354
tolR	TolR protein	STM0746	1.702	0.337	1.615	0.524
tolA	TolA protein	STM0747	2.219	0.211	1.508	0.465
htpX	Heat shock protein	STM1844	2.352	0.073	4.520	0.040
yebE	Conserved hypothetical protein	STM1880	6.237	0.053	5.186	1.99E-04
eco	Ecotin precursor	STM2262	2.677	0.135	4.064	0.002
	conserved hypothetical protein	STM3030	3.400	0.104	5.660	0.018
	Conserved hypothetical protein	STM3377	3.182	0.299	7.63	2.85E-04
	Putative membrane protein	STM3378	2.615	0.444	6.505	0.005
*ppiA**	peptidyl-prolyl cis-trans isomerase	STM3472	1.290	0.699	2.44	0.020
rpoH	RNA polymerase sigma-32 factor	STM3568	2.775	0.217	1.654	0.007
yihE	Conserved hypothetical protein	STM3996	2.780	0.219	1.799	0.070

Gene name	Gene function	Systematic name	Fold Induction WT vs WT*	t-test P-value	Fold induction CpxA* vs CpxA	t-test P-value
*dsbA**	thiol:disulfide interchange protein	STM3193	2.016	0.037	1.511	0.079
*cpxA**	two-component sensor kinase protein	STM4058	1.472	0.231	1.711	0.075
*cpxR**	two-component response regulatory protein	STM4059	1.725	0.414	2.735	0.015
*cpxP**	extracytoplasmic stress protein for protein-mediated toxicities	STM4060	9.732	0.049	8.154	1.41E-04
*psd**	phosphatidylserine decarboxylase proenzyme	STM4348	1.648	0.424	2.834	0.008
	putative aldehyde dehydrogenase (pseudogene)	STM4519	0.751	0.665	4.972	0.048

Table 11 –A selection of genes from Cpx Sanger array comparing WT vs WT induced and CpxA* vs CxpAΔ with a normalised ratio less than approximately 0.5. Data from this microarray experiment can be found in appendix A of this thesis. Previously known members of the Cpx regulon are asterixed (*).

Gene name	Gene function	Systematic name	Fold induction WT* vs WT	t-test P-value	Fold induction CpxA* vs CpxAΔ	t-test P-value
Crl	curlin genes transcriptional activator	STM0319	0.404	0.318	0.537	0.333
fimA	type-1 fimbrial protein, a chain precursor	STM0543	0.532	0.479	0.168	0.003
fimI	fimbrin-like protein FimI (pseudogene)	STM0544	1.057	0.756	0.449	0.082

Gene name	Gene function	Systematic name	Fold induction WT* vs WT	t-test P-value	Fold induction CpxA* vs CpxAΔ	t-test P-value
sigE	cell invasion protein	STM1090	1.109	0.844	0.361	0.146
sigD	cell invasion protein	STM1091	0.664	0.385	0.362	0.119
flgB	putative flagellar basal-body rod protein FlgB (proximal rod protein)	STM1174	0.499	0.288	0.693	0.137
sopE2	invasion-associated secreted protein	STM1855	1.022	0.933	0.41	0.251
sprB	possible AraC-family transcriptional regulator	STM2866	0.402	0.107	0.404	0.300
	hypothetical protein	STM2868	0.690	0.032	0.306	0.307
orgAa	oxygen-regulated invasion protein	STM2870	0.990	0.959	0.454	0.211
prgH	pathogenicity 1 island effector protein	STM2874	0.786	0.569	0.431	0.404
hilD	AraC-family transcriptional regulator	STM2875	0.510	0.021	0.130	0.264
sicP	chaperone (associated with virulence)	STM2879	0.685	0.130	0.287	0.045
sipF	probable acyl carrier protein	STM2881	0.675	0.387	0.133	0.288
sipC	pathogenicity island 1 effector protein	STM2884	0.685	0.547	0.453	0.489

Gene name	**Gene function**	**Systematic name**	**Fold induction WT* vs WT**	**t-test P-value**	**Fold induction CpxA* vs CpxAΔ**	**t-test P-value**
sipB	pathogenicity island 1 effector protein	STM2885	0.623	0.283	0.357	0.407
spaT	Unknown function	STM2886	0.646	0.121	0.391	0.478
spaP	secretory protein (associated with virulence)	STM2890	0.897	0.629	0.155	0.340
spaO	surface presentation of antigens protein (associated with type III secretion and virulence)	STM2891	0.681	0.080	0.158	0.336
ibpB	heat shock protein B	STM3808	0.435	0.496	0.525	0.014
*rseA**	sigma-E factor negative regulatory protein	STM2639	0.680	0.440	0.477	0.294

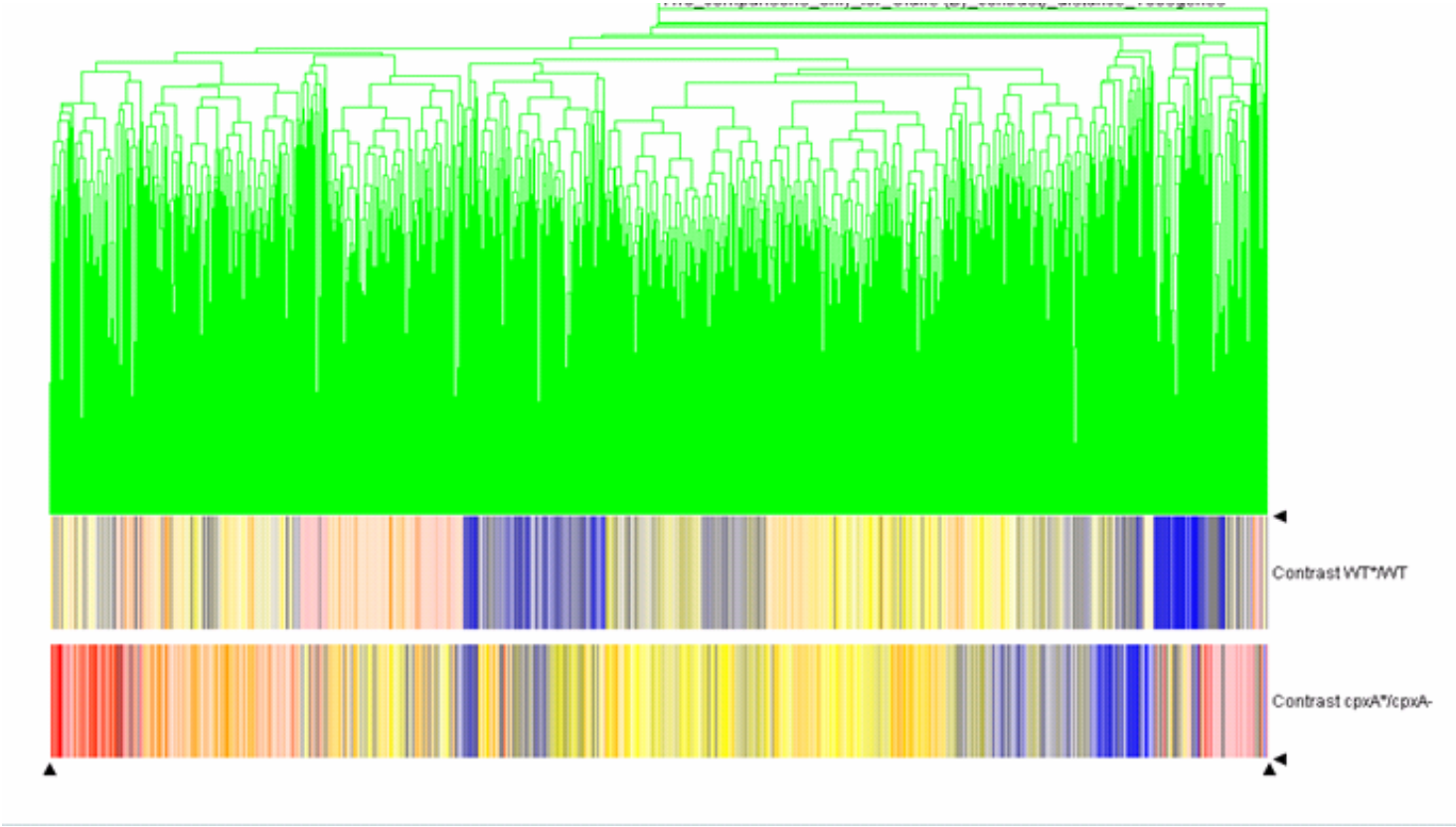


Figure 5.1 A genespring (Silicon Genetics) image depicting the *S*. Typhimurium array dataset comparing four Cpx associated strains allowing two comparisons of Cpx activitation vs. non-activation. A SL1344 CpxAΔ strain was compared against aSL1344 CpxA* (constitutively expressed) strain, and two WT SL1344 strains containing the nlpE inducible pND18 plasmid (either non-induced or induced with arabinose).
Data is coloured by expression levels with red lines being indicative of up-regulation of expression and blue indicative of repression.

5.4 CpxR-P binding site search

To investigate the regulation potential of any putative Cpx regulated genes, I performed a *S*. Typhimurium LT2 genome wide CpxR-P binding site search based on common CpxR promoter recognition sites. A Colibase (51) mismatch pattern search was performed using a specific nucleotide sequence based on promoter recognition by CpxR-P in *E. coli* (78) and also by searching upstream regions of known Cpx regulated genes in *S.* Typhimurium including *ppiA, cpxR* and *cpxP*. The coliBASE program was used to search the *S* Typhimurium LT2 genome for the potential Cpx binding motif GTAAA.

Using this CpxR-P recognition sequence, putative Cpx dependent promoters were identified for most of our genes identified as Cpx up-regulated including known Cpx dependent genes CpxR, CpxP and ppiA as expected. Of the 21 genes listed in table 10, a binding site could not be identified using the above motif for seven of these genes (*tolQ, htpX, yebE, ST*M3030, STM3378, *dsbA*, and *STM4519*) however it may be the case that there is no binding site present upstream of these genes and the increase in Cpx associated expression could be a secondary effect caused by other regulatory networks.

5.5 Quantitative Real-Time PCR

RT-PCR was performed as described in 2.11 for further study of the CpxAR system in *S.* Typhimurium and for confirmation of the Cpx status of several of our putatively Cpx regulated genes. An interesting set of genes shown to be up-regulated in our array included a set that appeared to be *Salmonella* specific. These four genes are named

STM3030, STM3377, STM3378 and *STM4519*. To check whether these genes had homologues in other bacteria, I performed a sequence comparison search in both coliBase and the NCBI BLAST site which searched all 923 completed microbial genomes on its database. Both these sequence searches failed to find any significant region of sequence homology between these genes and any of the other bacterial genomes apart from *Salmonella* species. As such, at this early stage I presume these genes may be *Salmonella* specific Cpx-regulated genes.

RNA was made from strains CpxA* SL1344 and CpxAΔ SL1344 as shown in 2.9 and oligonucleotides stm3030RTFor, stm3030RTRev, stm3377RTFor, stm3377RTRev, stm3378For, stm3378Rev, stm4519RTFor, and stm4519RTRev were designed for amplification of these four genes. As can be seen from Figure 5.2, the expression profiles of each of these 4 genes shows up-regulation in the induced CpxA* SL1344 strain vs. the CpxAΔ SL1344 strain which corresponds well with what is seen in our microarray experiments.

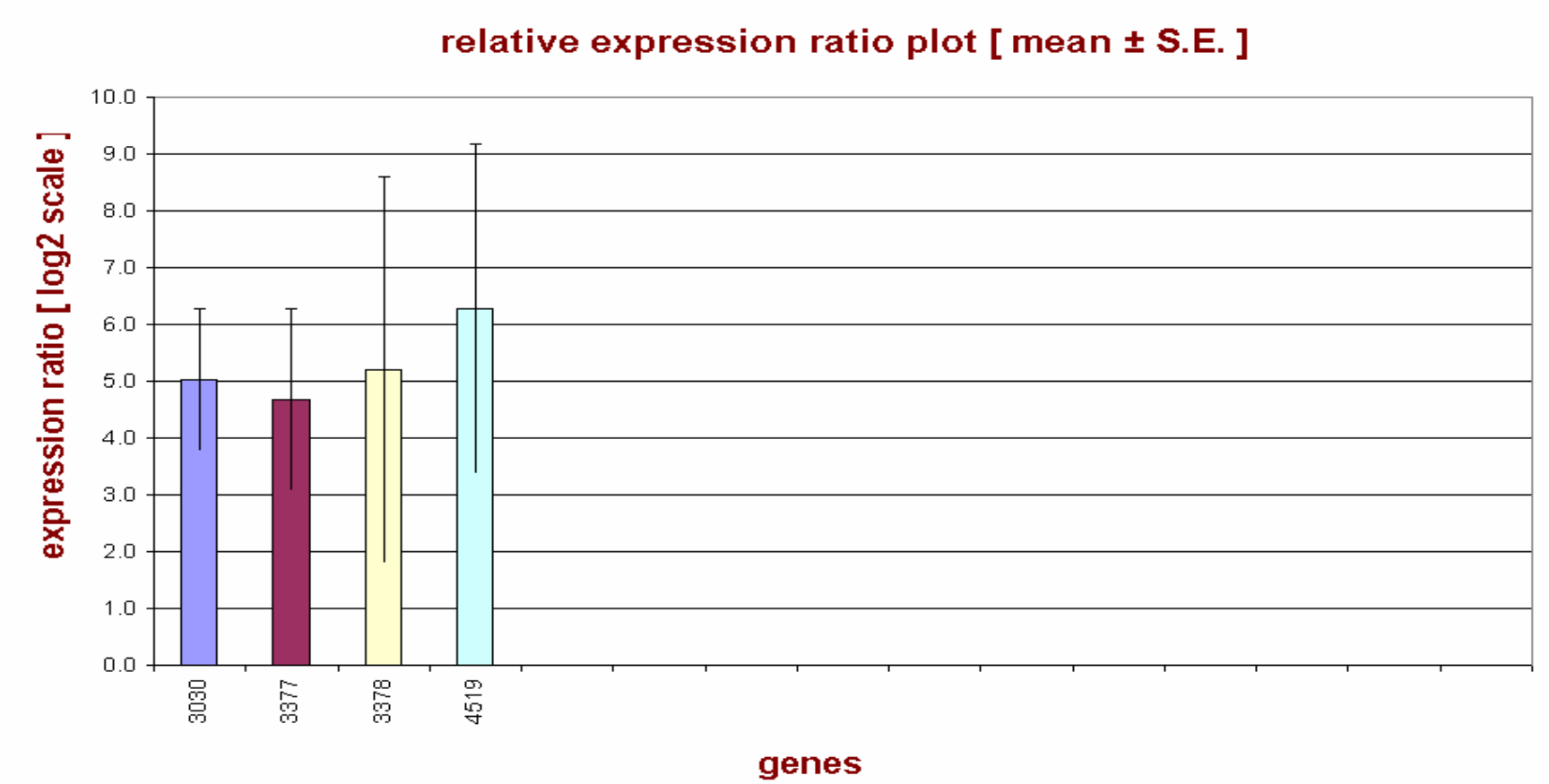


Figure 5.2 – Quantitative RT-PCR analysis of *Salmonella* specific genes *STM3030, 3377, 3378* and *4519* in constitutively expressing SL1344 CpxA* strain compared to SL1344 CpxAΔ.
REST RT-PCR profile obtained when looking at the relative expression of *Salmonella* specific genes in a Cpx induced *S.* Typhimurium strain compared to a CpxAΔ *S.* Typhimurium strain. lpp gene is an internal control

It was interesting to note that two fimbrial associated genes (*crl* and *fimA*) appeared to be down-regulated in our Cpx arrays when CpxA is induced. The Crl protein of *E. coli* interacts with σ^S to activate expression of genes such as *csgBAC* which encode the subunit of the curli proteins (325). Curli are a type of pili regulated by Cpx which are produced by *Salmonella* serovars as wells as *E. coli* and are involved in bacterial adhesion. *FimA* is part of the *fimAICDHF* operon in *S.* Typhimurium responsible for the biosynthesis of type 1 fimbriae. *S.* Typhimurium contains 13 fimbrial operons named *csg, fim, pef, lpf, bcf, saf, stb, stc, std, stf, sth, sti* and *stj*. To investigate further the down regulation of *crl* and *fimA* seen in our Cpx array, RNA was made from strains CpxA* SL1344 and CpxAΔ SL1344 as shown in 2.9 and oligonucleotides csgARTFor, csgARTRev, fimARTFor, fimARTRev, sthARTFor, sthARTRev, stbARTFor, stbARTRev, bcfARTFor, bcfARTRev, stcARTFor, stcARTRev, lpfARTFor and lpfARTRev were designed for amplification of *csgA*, *fimA* and several other known fimbrial genes (*sthA, stbA, bcfA, stcA* and *lpfA*). As is shown in Figure 5.4, all 7 of these fimbrial genes appear to be down-regulated in the induced CpxA* SL1344 strain vs. the CpxAΔ SL1344 strain which corresponds well with what is seen in our microarray experiments.

5.6 Analysis of SPI-1 associated genes

Study of down-regulated genes on our Cpx microarray highlighted the presence of many *Salmonella* pathogenicity island 1 (SPI-1) associated genes. SPI-1, encoding type III secretion system -1 (TTSS-1), mediates invasion of epithelial cells by *S.* Typhimurium (116). The SPI-1 TTSS genes are positively regulated by HilA and

previous work has shown *hilA* expression at pH 6.0 to be dependent on CpxA in *S.* Typhimurium. This would not appear to be the case at pH8.0, but it would appear that the Cpx system has a slightly negative effect of *S.* Typhimurium invasion at high pH (165, 268).

Figure 5.3 shows the genetic organisation of SPI-1

Table 12 below shows the SPI-1 associated genes found to be down-regulated in our microarray.

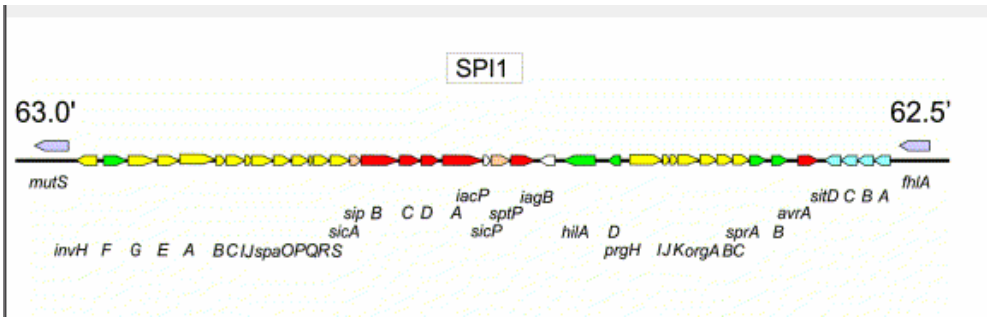


Figure 5.3 - Genetic organisation of SPI-1. Reproduced from Hansen-Wester (142)

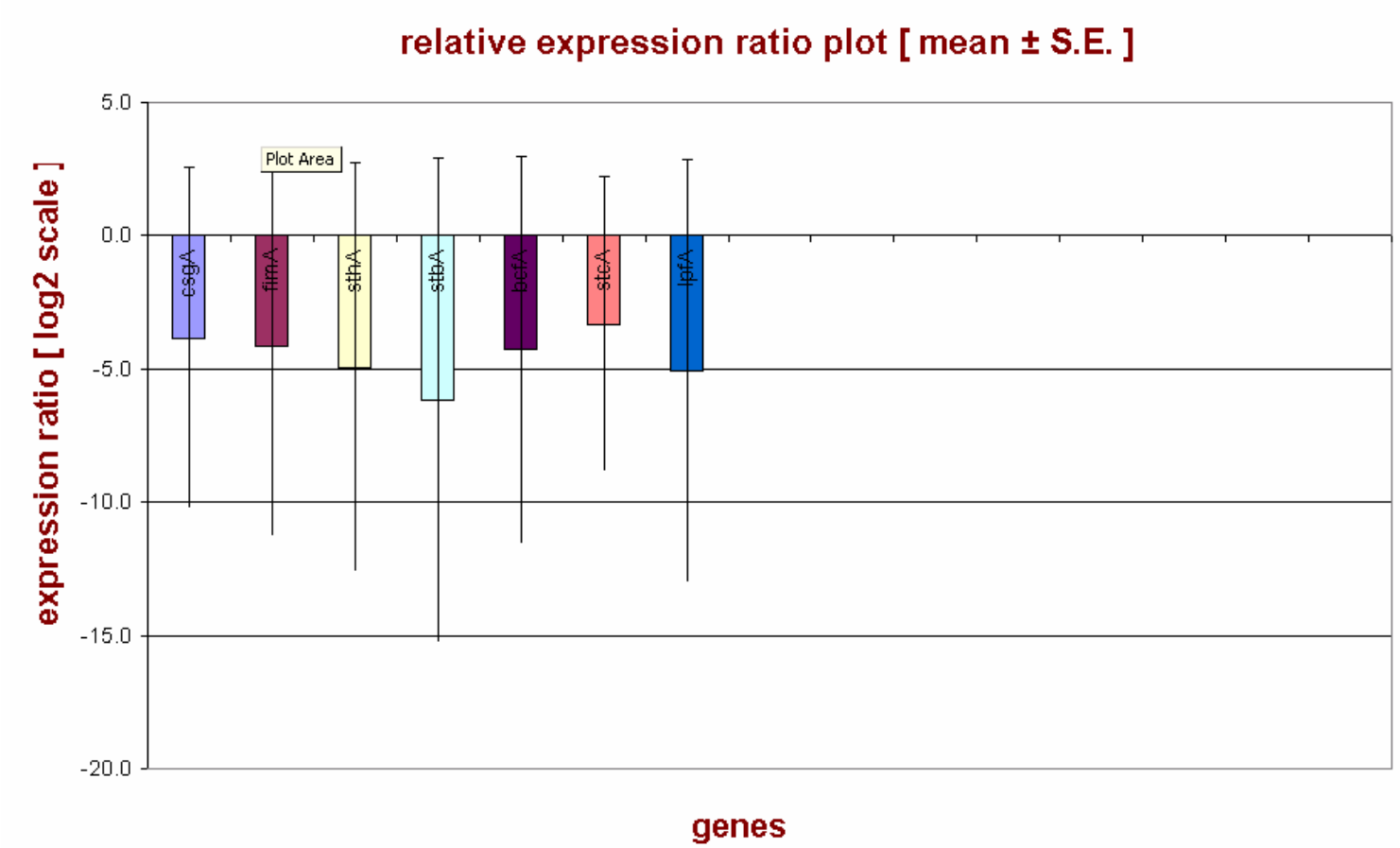


Figure 5.4 – Quantitative RT-PCR analysis of *Salmonella* fimbrial genes *csgA, fimA, sthA, stbA, bcfA, stcA* and *lpfA* in constitutively expressing SL1344 CpxA* strain compared to SL1344 CpxAΔ. REST RT-PCR profile obtained when looking at the relative expression of *Salmonella* fimbrial genes in a Cpx induced *S.* Typhimurium strain compared to a CpxAΔ *S.* Typhimurium strain. lpp gene is an internal control.

Table 12 – A list of all SPI-1 associated genes from the Cpx Sanger array with a normalised ratio less than 0.5 in either WT induced vs non-induced or CpxA* vs CpxAΔ.

Gene name	**Systemic name**	**Proposed role in SPI-1**
pipC	STM1090	Pathogenicity island-1 encoded protein
sopB	STM1091	Cell invasion protein
sopE2	STM1855	Invasion associated secreted protein
	STM2868	Hypothetical protein
orgAa	STM2870	oxygen-regulated invasion protein
sprB	STM2866	Possible AraC-family transcriptional regulator
prgH	STM2874	Needle complex inner membrane protein
hilD	STM2875	AraC-family transcriptional regulator
sicP	STM2879	Chaperone (associated with virulence)
sipF	STM2881	Probable acyl carrier protein
sipC	STM2884	Pathogenicity island-1 effector protein
sipB	STM2885	Pathogenicity island-1 effector protein
spaT	STM2886	Unknown function
spaP	STM2890	Secretory protein (virulence associated)

Gene name	Systemic name	Proposed role in SPI-1
spaO	STM2891	surface presentation of antigens protein (associated with type III secretion and virulence)
avrA	STM2865	Secreted effector protein

To investigate the possible effect of activation of the CpxAR system on expression of SPI-1 genes, a western blot was set up as described in 2.6 to compare the expression levels of SipC (a pathogenicity island-1 effector protein) in both SL1344 WT and also a constitutively expressing SL1344 CpxA* strain. As can be seen in Figure 5.5, expression of SipC is much reduced in the SL1344 CpxA* strain compared to SL1344 WT. This verifies the microarray findings. This experiment was repeated in triplicate and the same results were achieved on each occasion.

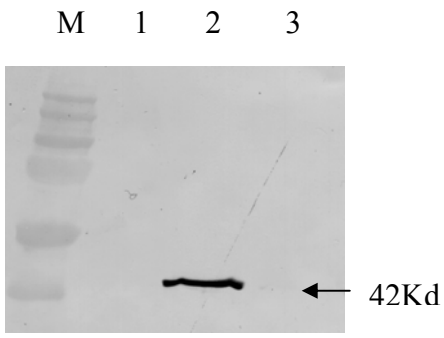


Figure 5.5 - Analysis of sipC expression in SL1344 WT and CpxA* by western blot. M is BioRad Precision Plus Protein standard, 1 is negative control, 2 is SL1344 WT and 3 is SL1344 CpxA*.

5.7 Discussion

Following characterisation of the RpoE and PSP ESR systems I carried out studies to characterise the CpxAR ESR of *S.* Typhimurium.

Microarray technology has been useful in the identification of bacterial virulence genes and as such a microarray experiment was performed to identify possible Cpx regulated genes with the aim of identifying new genes involved in the *Salmonella* Cpx ESR.

Analysis of the array data identified many genes up- or down-regulated and my first aim was to compile a table of up- and down-regulated genes (Appendix A). Given more time I would like to have been able to repeat the microarray experiment as the criteria for gene selection in appendix A was less stringent than it could have been. This less stringent approach was used to include known Cpx regulated genes such as *htrA* and as such caution should be taken when analysing the data in isolation. From the large number of genes identified (appendix A) a more concise list of genes was compiled which included genes known to be Cpx regulated in *E. coli* and genes selected for further investigation in this chapter (Table 10 and 11). A CpxR-P binding site consensus search was performed to look for the presence of any potential Cpx regulated promoters within a reasonable distance of our putatively Cpx regulated genes. This identified binding sites upstream of the known Cpx regulated genes *ppiA, cpxR* and *cpxP* and also putative Cpx binding sites upstream of many of the genes which were identified in our array as potentially Cpx up-regulated. Little is known about the role of many of these genes in the Cpx system of *S.* Typhimurium so future work will be to construct deletion mutations of these genes and investigate more

thoroughly their role in the Cpx transduction system and potentially any role they may have in the virulence of *S.* Typhimurium.

I identified the following groups of genes of particular interest:

1. STM3030, STM3377, STM3378 and STM4519 are possible *Salmonella*-specific genes that appear to be Cpx up-regulated.

2. Fimbrial associated genes (*crl* and *fimA*) appeared to be Cpx down-regulated

3. Many *Salmonella* pathogenicity island 1 (SPI-1) TTSS structural and effector genes appear to be Cpx down-regulated.

4. The *tol* genes *tolQ, R* and *A* appear to be Cpx up-regulated.

As the *tol* genes also appear to be RpoE regulated, these will be discussed in more detail in chapter 6 which discusses overlaps between the RpoE and Cpx systems.

To investigate further if the genes STM3030, STM3377, STM3378 and STM4519 are positively up-regulated by Cpx, RT-PCR was carried out as shown in Figure 5.2 which confirmed the increase in expression observed in our microarray. In addition to this, I performed sequence homology searches which support my hypothesis that these four genes may be *Salmonella* specific. It would be interesting to investigate these genes further and their role in the Cpx response of *Salmonella*. In particular it would be interesting to see if these genes are involved in the virulence and stress response of *Salmonella*.

My identification of fimbrial associated genes in *S.* Typhimurium being Cpx regulated is in agreement with what has been found in *E.coli*.

Cpx is involved in the production of P (Pap) pili which are important virulence determinant of uropathogenic *E.coli*. The published studies give conflicting results. In the first study CpxAR was reported to positively affect production of P pili directly and indirectly (169). In the absence of glucose in the culture medium, phosphorylated CpxR (CpxR-P) binds upstream of the *pap* genes to activate their expression (169). CpxAR also regulates the expression of proteins such as DsbA and PpiA, which assist in pilin assembly in the periplasm and the P pili produced by a *cpxR* mutant are shorter than those of WT *E.coli* (169).

In the second study, Cpx was found to negatively affect P pili production (155). P pili exhibit environmentally-responsive phase variation switching between OFF and ON phases. The transition between OFF and ON phases is mediated by the Leucine-responsive regulatory protein (Lrp) (155). Activation of CpxAR interferes with transition from phase OFF to phase ON by competing with Lrp for DNA binding upstream of the *pap* gene cluster (155). It was suggested that the difference in the findings was due to a multicopy plasmid encoding the activator genes *papI* and *papB* used in the first study, which interferes with normal phase variation frequencies (155).

Cpx was shown to positively regulate expression of a different class of pili, the type IV bundle-forming pili (BFP), of enteropathogenic E. coli (EPEC). An EPEC *cpxR* mutant produced less BFP and was less able to adhere to eucaryotic cells *in vitro* (269). Cpx also affects the production of a third type of pili, Curli. Curli are produced by *Salmonella sp.* as well as *E.coli* and are involved in adhesion of bacteria to abiotic surfaces, eucaryotic cells and biofilm formation. The genes for curli production are

grouped into two adjacent, divergently transcribed operons, *csgAB* and *csgDEFG*. CsgD is a transcriptional activator of the *csgAB* operon that encodes the Curli subunit CsgA and the nucleator protein CsgB (125). CsgD also activates other genes involved in biofilm formation (125). The expression of Curli is complex and is affected by a number of DNA binding proteins (186, 329, 377). Cpx negatively regulates Curli production. CpxR-P binds upstream of *csgD* inhibiting the binding of the activator OmpR (186). High osmolarity inhibits Curli production (186). Cpx-mediated inhibition of *csgD* expression was activated in high salt media. However, repression of Curli production in the presence of high concentrations of sucrose was not mediated by Cpx but rather H-NS (186).

Finally, a functional CpxAR pathway is important for surface sensing and adhesion to inanimate surfaces by *E.coli* (282). It was postulated by the authors that the role of the CpxAR pathway was to regulate genes required to maintain a stable cell-surface contact.

The curli fibres are highly conserved between *S.* Typhimurium and *E. coli* (329). It is not surprising therefore that in my microarray studies *crl* (and *fimA*) are down regulated when the Cpx system is activated. In addition to this, my RT-PCR studies have shown that 7 fimbrial genes appear to be down regulated when the Cpx system is activated. This group of fimbrial genes was chosen for study as many of them have been shown to be required for intestinal persistence in mice in *S.* Typhimurium (391). It is encouraging that under the conditions tested, our RT-PCR studies allowed detection of the expression levels of these fimbrial genes as past studies have suggested that the majority of serotype Typhimurium fimbrial genes are poorly

expressed *in vitro*, while their expression is induced *in vivo* (168). This additional RT-PCR data has proved crucial as many of the fimbrial genes investigated above were missing from our original microarray data experiment.

Salmonella has two type III secretion systems (TTSS-1 and TTSS-2) which are encoded on two areas of the *Salmonella* chromosome referred to as *Salmonella* pathogenicity island -1 and *Salmonella* pathogenicity island -2 (142). As mentioned earlier, many SPI-1 proteins and effectors appear to be down-regulated by the Cpx signal transduction system as shown by data in my Cpx microarray experiment. Contrary to this, none of the SPI-2 encoded genes or effectors appear to be up- or down-regulated in our array so possible future work could be to repeat the above microarray experiments using RNA from cells grown under SPI-2 inducing conditions. In this way we could see if there is any difference in the expression of SPI-2 genes and effectors in response to the Cpx system when under SPI-2 inducing conditions.

Salmonella uses the TTSS to transport effector proteins into the host cell, playing a pivotal role in *Salmonella* cell to host cell interaction. TTSS-1 is required for initiating intestinal inflammation (143, 384) and invasion of epithelial cells (116) while TTSS-2 is essential for systemic infection (29) and intracellular growth (219).

All genes within table 12 are located in centisome 63 TTSS-1, apart from *pipC, SopE2* and *sopB* which are TTSS effectors required for invasion into host cells (250, 405) located further upstream in the genome.

To investigate the possible effect of activation of the CpxAR system on expression of SPI-1 genes, a western blot was performed to compare the expression levels of SipC (a pathogenicity island-1 effector protein) to study the possible effect of activation of the CpxAR system on expression of SPI-1 genes and to help verify what was seen in our microarray experiments. Results showed the levels of SipC are much reduced in a CpxA* strain compared to the WT strain correlating with our array data.

Do my findings on the down-regulation of fimbrial and SPI-1 gene expression have any functional significance to *S.* Typhimurium infection? Previous work in my laboratory showed that that a *S.* Typhimurium *cpxA** mutant is less invasive than WT and *S.* Typhimurium *cpxAΔ* strains (165). Further evidence of a role for the Cpx system in attachment and invasion came from studies showing a Tn*phoA* transposon insertion in CpxA reduces the ability of *S. Typhi* to adhere to and invade epithelial cells *in vitro* (206). My results indicate that this could be due to down-regulation of SPI-1 genes and / or reduced fimbrial expression.

Although the suggested primary role of the CpxAR system in laboratory *E. coli* strains is to sense envelope protein mis-folding and up-regulate the expression of protein folding and degradation factors, laboratory *E. coli* strains do not produce many pili or TTSS. The above data shows that in *S.* Typhimurium, the CpxAR system negatively regulates the expression of many TTSS-1 proteins and effectors and fimbriae. Therefore, one of the roles of the Cpx system is to ensure that the production of TTSS and fimbriae are only produced when they are needed. This may

be to conserve energy as both TTSS and fimbriae are large macromolecular structures and their production is very energy consuming. Alternatively, the Cpx system may shut down synthesis of TTSS and fimbriae to aid shedding of *S.* Typhimurium which help the spread of *S.* Typhimurium.

Work is continuing within our lab looking at the differences in the proteins in the periplasmic inner and outer membrane fractions between CpxA* and CpxAΔ deletion mutants. Preliminary data from these studies has shown that there appears to be a group of proteins present in the periplasmic inner membrane fraction of CpxA* that is not present in CpxAΔ. These proteins are in the process of being identified and it will be interesting to see if any of these proteins correspond with what is seen in our microarray studies.

Future work within the lab will hopefully allow some of the Cpx regulated genes identified in my study to be characterised more fully. Also it is hoped that the role and importance of the CpxAR system in the down regulation of SPI-1 and fimbrial genes can be further elucidated.

Chapter Six – Overlap of RpoE and Cpx extracytoplasmic stress response systems in *S.* Typhimurium

6.1 Introduction

Throughout this thesis I have sought to study two of the well known extracytoplasmic stress response systems of *S.* Typhimurium: The RpoE ESR and the two-component CpxAR signal transduction ESR.

Following investigation of data from our CpxAR microarray experiment, analysing the expression of genes in *S.* Typhimuriu*m* under activation and non-activation of the CpxAR system, there were several genes that appeared to be regulated by both the CpxAR system and also the RpoE system, as identified by microarray analysis by G. Rowley (331). A list of some of these genes is shown in table 13 below.

Table 13 – A list of genes from the Cpx Sanger arrays and G. Rowleys RpoE microarray with a normalised ratio greater or approximately equal to 2. Data shown in third and fourth columns is from the Cpx microarray comparing WT vs WT induced direct ratios and CpxA* vs CxpAΔ direct ratios. Data shown in fifth column is from RpoE microarray comparing an overexpressing RpoE SL1344 strain with WT SL1344. Entire data from the Cpx microarray experiment can be found in appendices A of this thesis

Gene name	**Gene function**	**Fold induction WT* vs WT**	**Fold induction CpxA* vs CpxA-**	**Fold induction RpoE+ vs WT**
htrA	heat shock protein HtrA	2.44	3.14	32.75
rpoH	RNA polymerase sigma-32 factor	2.78	1.65	7.19
psd	Phosphatidylserine decarboxylase proenzyme	1.65	2.83	5.10
tolR	tolR protein	1.7	1.62	4.29
pal	peptidoglycan-associated lipoprotein precursor	1.05	1.83	2.79
htpX	heat shock protein	2.35	4.52	2.26
yjfN	hypothetical protein	0.73	3.52	1.87
ompX	outer membrane protein x precursor	2.25	2.25	1.79

Both the CpxAR and RpoE systems are extracytoplasmic stress responses which sense and respond to mis-folded proteins in the bacterial envelope however their activating signals and signal transduction mechanisms can be very distinct.

The RpoE system responds to OMP mis-folding brought on for example by high temperatures and ethanol stress by disruption of the RseA – RseB complex via degradation of RseA by DegS allowing the release of σ^E into the cytoplasm where it interacts with RNA polymerase core enzyme to induce transcription of factors involved in OMP folding and degradation such as *htrA* and *fkpA* (54, 63, 94, 330). The Cpx system responds to different environmental conditions such as increased pH (64) and NlpE overproduction (350) by release of CpxP inhibition activating phosphotransfer between CpxA and CpxR (314). Phosphorylated CpxR initiates transcription of genes involved in envelope protein folding and degradation such as *dsbA, ppi* and *htrA*. In this way, we can see that although these two systems operate in a different manner, and are induced by different signals, they both can result in the increased transcription of *htrA* (whose role in *S.* Typhimurium is investigated further in Chapter 7) for example.

As such, I decided to investigate a group of genes, the *tol* genes, which appeared to be regulated by both the RpoE and CpxAR stress response systems. The *tol* group of genes are split into two convergently transcribed operons, *tolQRA* and *tolBpalybgF*. *tolR* was identified as a σ^E regulated gene in *S.* Typhimurium following the two plasmid screen performed by colleagues within our lab. *tol* genes *tolR, tolB* and *pal* were also seen to be up-regulated in the over-expressing RpoE microarray and S1 mapping and consensus searching identified a putative RpoE dependent promoter within the CDS of *tolQ* (349). In addition to this, *tolQRA* were all found to be up-

regulated in our Cpx microarray. It is thought that as these genes are involved in both these ESR systems, they may play a crucial role in the virulence of *S.* Typhimurium.

6.2.1 Tol-Pal proteins

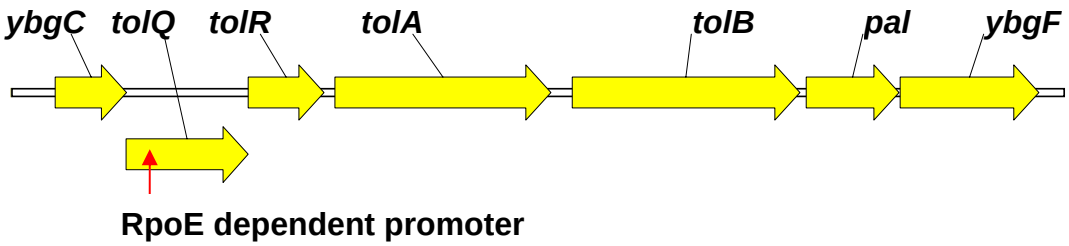


Figure 6.1 Operon structures of *tol-pal* genes

The *tol* genes can be described as two convergently transcribed operons named *tolQRA* and *tolBpalybgF* as shown in Figure 6.1. It is no surprise that the *tol* genes are involved in the ESR systems of *S.* Typhimurium, as these genes are known to play a role in maintaining outer membrane integrity (205). In *E. coli*, mutations in any of the *tol-pal* genes have resulted in hypersensitivity to bile salts and detergents such as sodium cholate and SDS and also capsule synthesis induction leading to the development of a mucoid phenotype (205). The Tol proteins of *E. coli* have also been found to be involved in the translocation of group A colicins which are bacterial protein toxins active against *E. coli* and other bacteria. These group A colicins can kill cells by depolarizing the cytoplasmic membrane and they can have cytotoxic activity against cytoplasmic nucleic acids (204).

There is a high degree of conservation of the tol-pal system in other bacteria, with the Tol-Pal proteins of *Erwinia chrysanthemi* showing high levels of homology with *E.*

coli. *E. chrysanthemi tol-pal* mutants showed a hypersensitivity to bile salts, and were shown to affect cell morphology and virulence (86). It is also known that the Tol-Pal pathway is essential to cell viability in both *P. aeruginosa* (81), *E. coli* 0157 (122) and *Haemophilus ducreyi* (356).

Although the *S.* Typhimurium *tol* genes have been shown to play a role in bile resistance, there are some differences in the effect of Tol-Pal proteins on *E. coli* and *S.* Typhimurium. Mutants in the Tol-Pal pathway of *S.* Typhimurium do not appear to result in a mucoid phenotype or exhibit growth defects compared to WT. Also, the RcsCB system does not appear to regulate the transcription of the *tol* genes in *S.* Typhimurium as it does in *E. coli* (306).

tolR was identified as being a member of the RpoE regulon in *S.* Typhimurium through the identification of an RpoE dependent promoter located in the coding region of the *tolQ* gene (349).

A *tolB* mutant has previously been shown to demonstrate a defect in virulence compared to WT *S.* Typhimurium when administered orally to mice (366) and colleagues within our lab recently established that *tolR* also plays an important role in the pathogenesis of *S.* Typhimurium in a murine host (331).

As the Tol-Pal pathway appeared to be both RpoE and CpxAR regulated, as exhibited by microarray analysis, (table 13), and also appeared to play an important role in the virulence of *S.* Typhimurium, I chose to study further the Tol-pal pathway. My initial studies began with analysis by quantitative RT-PCR. Colleagues within the lab had previously constructed a *tolR* deletion mutant and to investigate this pathway in more detail I constructed a single deletion mutant lacking the *tolB* gene. In addition I constructed deletion mutations lacking the entire *tolQRA* operon, and also the entire

tolBpalYbgF operon. Extensive phenotypic and *in vivo* characterisation was then performed. Final studies investigated the vaccine potential of the *tol-pal* mutants.

6.2.2 Quantitative Real-Time PCR

RT-PCR was performed as shown in 2.11 for further study of the expression of Tol – Pal proteins in *S.* Typhimurium.

RNA was made from strains SL1344 CpxA* and SL1344 CpxAΔ SL1344 as shown in 2.9 and oligonucleotides TolQRTfor, TolQRTrev, TolBRTfor and TolBRTrev were designed for amplification of TolQ and TolB, as these two genes are the first genes of the two Tol – Pal operons. As can be seen from Figure 6.2 the expression profiles of TolQ and TolB show slight up-regulation in the induced SL1344 CpxA* strain vs. the SL1344 CpxAΔ SL1344 strain which corresponds with what was seen in our microarray experiments.

The up-regulation observed however was quite small and this experiment needs further validation as these initial RT-PCR experiments were only carried out on one occasion. Similar RT-PCR data is also required to study the expression of these *tol – pal* genes under RpoE inducing conditions, to further support the microarray data and verify my hypothesis that these genes are both RpoE and Cpx regulated.

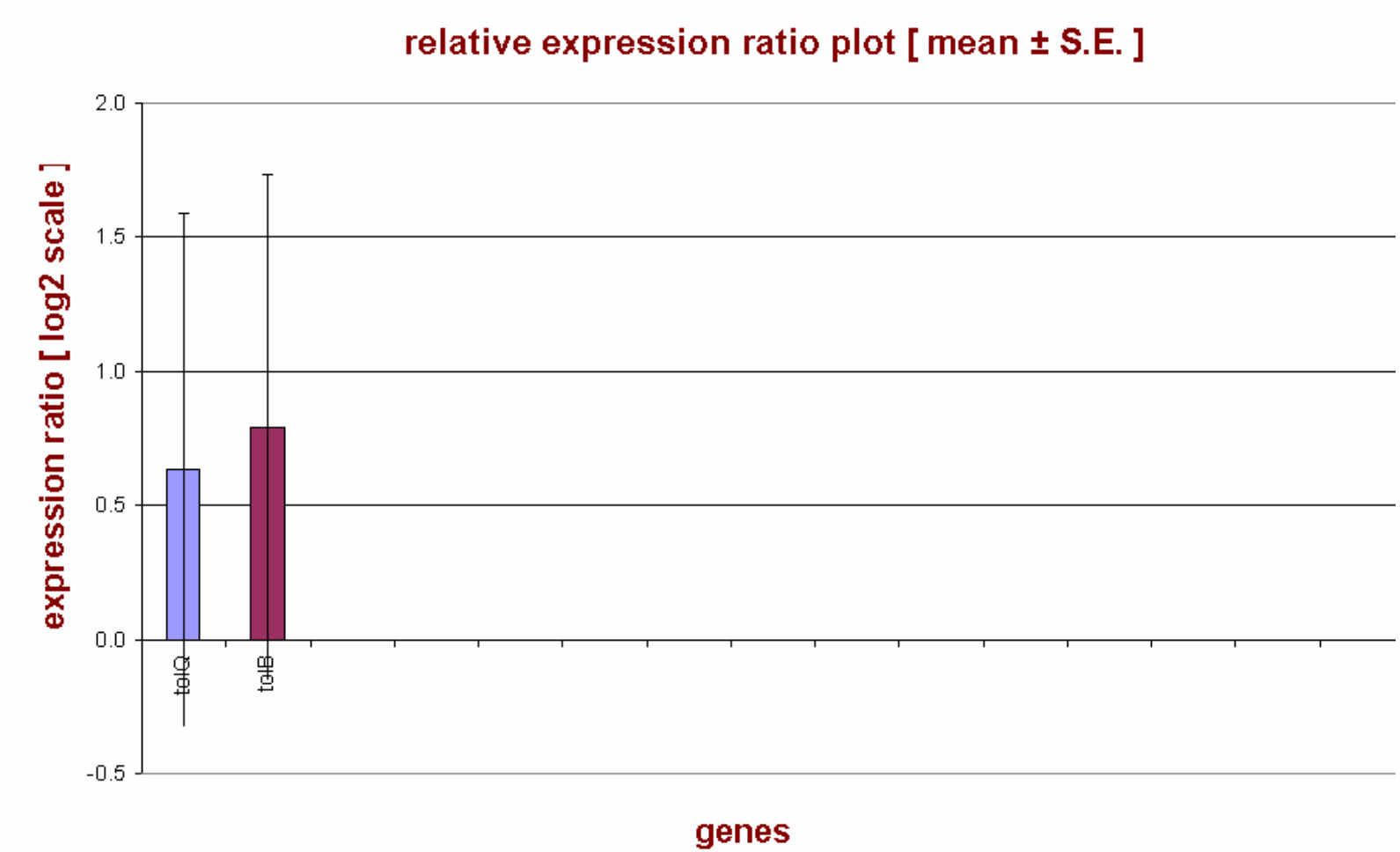


Figure 6.2 - REST (relative expression software tool) displaying relative expression ratio plot. Expression levels of genes *tolQ* and *tolB* from induced SL1344 CpxA* RNA compared to RNA from a SL1344 CpxAΔ strain. *lpp* gene is an internal control

6.2.3 Mutagenesis of *tol-pal* genes

RED mutagenesis was used to construct a single *tolB* deletion mutant, and also *tolQRA,* and *tolBpalYbgF* deletion mutants in *S.* Typhimurium. Oligonucleotides tolQRAredFOR, tolQRAredREV, tolBpalybgFredFOR, tolBpalybgFredREV, and tolBREDfor were designed as shown in table 4 for construction of these deletion mutants. Oligonucleotides TolBextFor and ybgFextRev2 were designed and used to verify the efficient mutagenesis of both the single *tolB* deletion mutant and also the *tolBpalybgF* deletion mutant. Oligonucleotides TolQRAextFOR and TolQRAextREV were designed and used to verify the efficient mutagenesis of the *tolQRA* deletion mutant, and also to double check the existing *tolR* single deletion mutant. Table 14 below shows a list of all *tol* mutants used in this study.

Table 14 – A list of *tol* mutants used in this study

Strain	**Genotype**	**Source**
GVB1360	SL1344 *ΔtolR*	G.Rowley (331)
GVB1845	SL1344 *ΔtolQRA*	This study
GVB1839	SL1344 *ΔtolB*	This study
GVB1840	SL1344 *ΔtoBpalybgF*	This study

6.2.4 *In vitro* analysis of *tol - pal* genes.

Following construction of *tol – pal* deletion mutations in *S.* Typhimurium, an array of phenotypic assays were established to investigate the impact that loss of any of these genes may have on the *in vitro* behaviour of these strains. Previous work within our laboratory investigating the SL1344 *tolR* deletion strain, showed this gene to have no effect upon exposure to Polymyxin B, hydrogen peroxide or an array of antibiotics. It did not appear to have a mucoid colony phenotype (as is seen with *E. coli*) nor did it appear to exhibit any temperature sensitivity when compared with WT SL1344.

Initial disc diffusion assays were set up to study the effect of the antimicrobial compounds Polymyxin B (300 units in 10µl), Fusidic acid (10µg), and Novobiocin (30µg). All *tol – pal* deletion mutations showed no difference in the zone of inhibition compared to WT (Data not shown).

An inhibition assay was set up to study the effect of our *tol – pal* mutants on SDS, as shown in Figure 6.3. As can be seen in Figure 6.3 at all concentrations of SDS tested (2%, 4% and 6%) all 4 *tol – pal* mutants exhibited much greater inhibition than is seen in the WT. At all concentrations of SDS tested, statistical analysis using ANOVA showed all four *tol* mutants to be considered extremely significantly different to WT, with $p<0.001$ in all cases. The greatest effect is seen in the mutant *tolR* and *tolQRA* deletion strains which experience greatly reduced survival in 2% SDS, and cannot grow in the presence of 4% or 6% SDS. The deletion mutants *tolB* and *tolBpalybgF* survive slightly better in SDS at 2%, but still greatly reduced in comparison to WT. Both *tolB* and *tolBpalybgF* mutants experience ever greater inhibition at 4% and 6% SDS compared to WT, though these strains are able to grow at these concentrations.

As *tol – pal* mutants have been shown to have increased hypersensitivity to bile acids in *E. coli* and *E. chrysanthemi,* inhibition assays were set up to investigate the response of our *tol – pal* mutants to the bile acids sodium cholate, and deoxycholic acid. As can be seen in Figure 6.4 our *tol – pal* mutations all exhibit a great increase in inhibition upon exposure to 2% Sodium cholate compared to WT. 4 and 6% sodium cholate proved toxic to all four of our *tol – pal* mutations but not for the WT strains. At all concentrations of sodium cholate tested, ANOVA showed all four Tol mutants to be considered extremely significantly different to WT, with $p<0.001$ in all cases.

Figure 6.5 shows the response of our *tol – pal* mutations to varying concentrations of deoxycholic acid, however concentrations of 0.2, 0.4 and 0.6% proved toxic to all 4 of our Tol – Pal strains. SL1344 WT managed to grow at all of these concentrations of deoxycholic acid. At all concentrations of deoxycholic acid tested ANOVA showed all four *tol* mutants to be considered extremely significantly different to WT, with $p<0.001$ in all cases. Taken together these results show that *S.* Typhimurium *tol – pal* mutants also exhibit the increased hypersensitivity to bile acids as seen in other bacteria.

Following the above studies looking at bile acids, similar inhibition assays were set up to look at the effect that the protonophore CCCP has on our *tol – pal* mutations. CCCP has been shown to affect an *rpoEΔpspAΔ* double mutant (17) and was found in my earlier studies to affect a SL1344 *smpAΔ* strain.

As can be seen in Figure 6.6, all four of my *tol – pal* mutations exhibit levels of inhibition to CCCP when present at 2, 4 and 8 micrograms per ml, however it is only at 8 micrograms per ml we observe a significant different between the response of our *tol – pal* mutations and the SL1344 WT strain. At this higher concentration, CCCP proves almost toxic to the *tol – pal* deletion strains, while WT can survive much

better. At all concentrations of CCCP tested, ANOVA showed all four *tol* mutants to be considered extremely significantly different to WT, with $p<0.001$ in all cases.

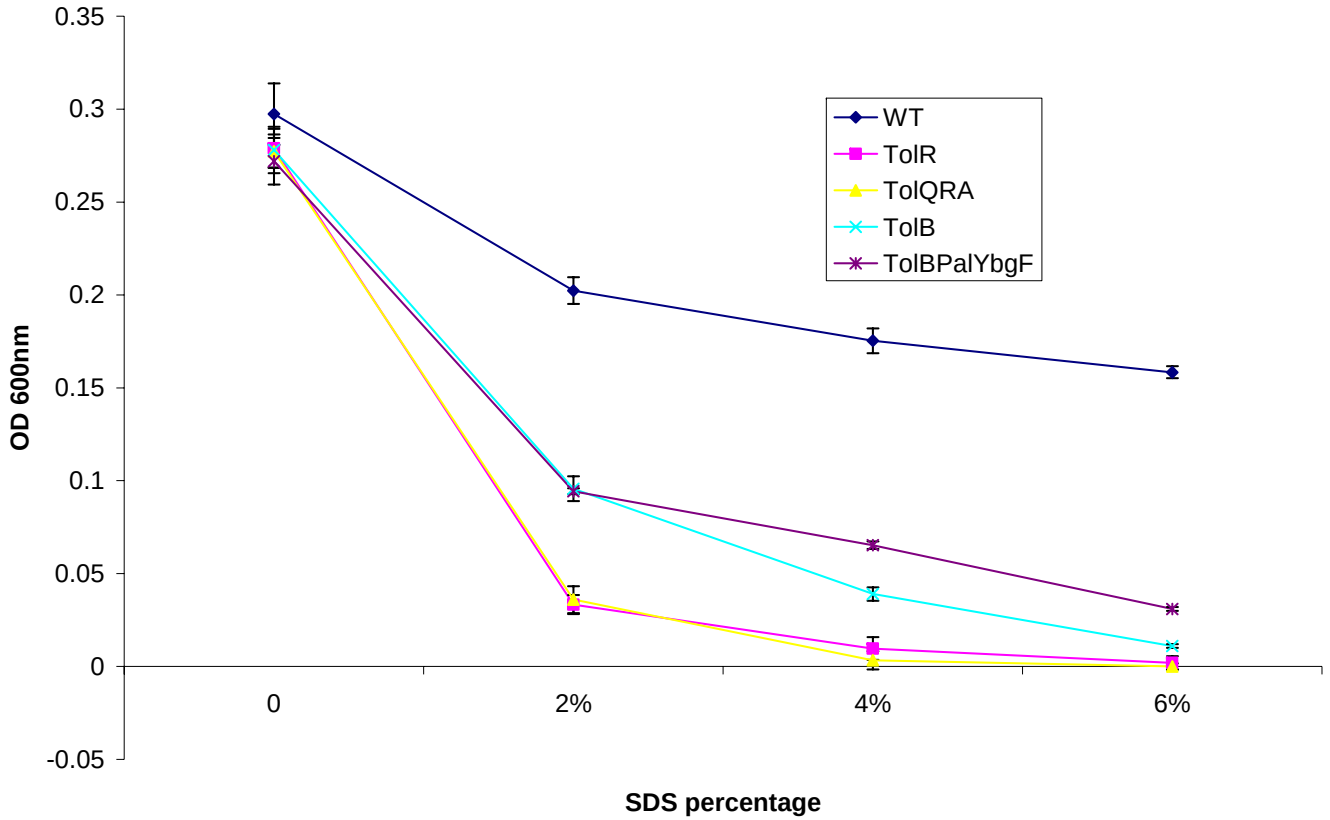


Figure6.3 - Effect of *tol – pal* mutations on the growth of *S.* Typhimurium in liquid media supplemented with SDS
The *tolR, tolQRA, tolB* and *tolBpalybgF* deletion mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 2, 4 and 6% SDS at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm Experiments were performed in triplicate and the error bar indicates the standard deviation.

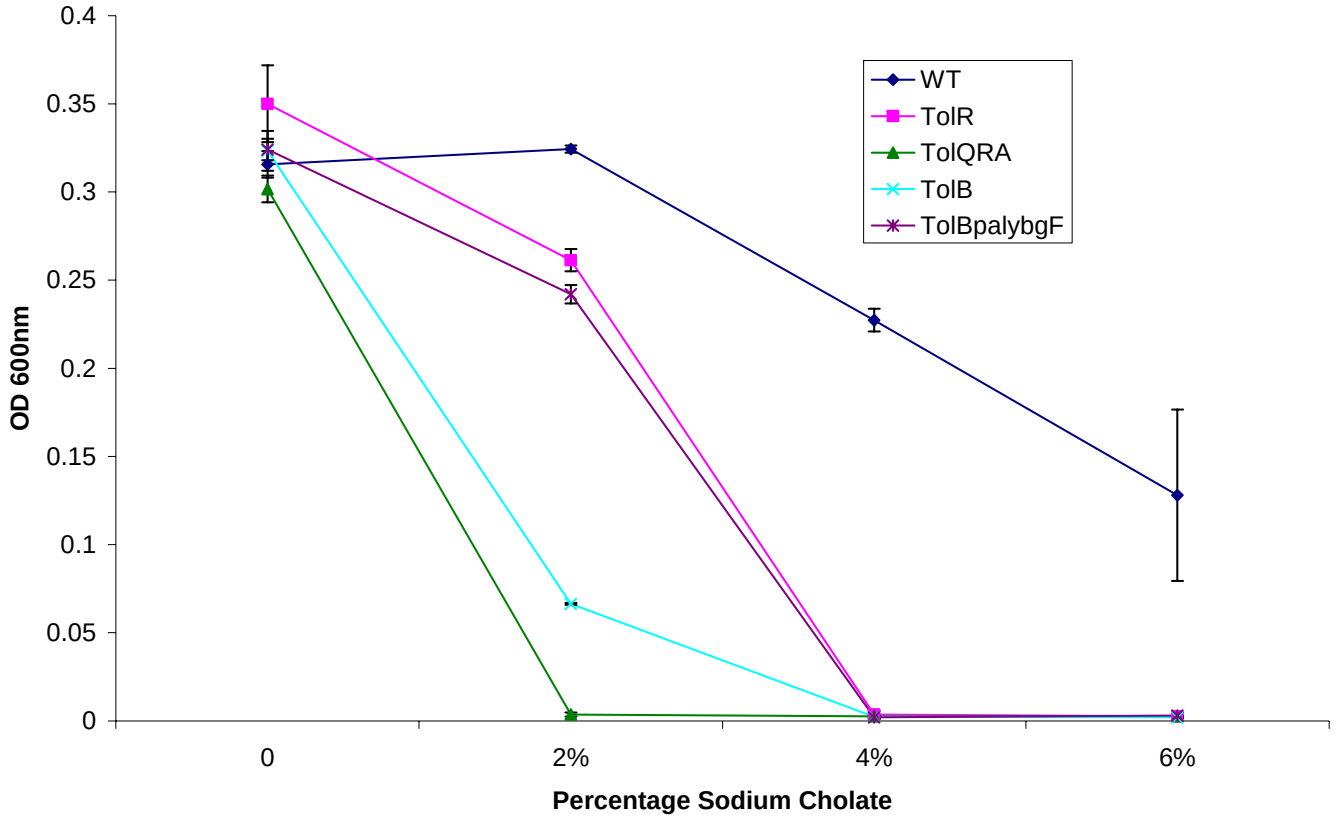


Figure 6.4 - Effect of *tol – pal* mutations on the growth of *S.* Typhimurium in liquid media supplemented with sodium cholate
The *tolR, tolQRA, tolB* and *tolBpalybgF* deletion mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 2, 4 and 6% sodium cholate at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm Experiments were performed in triplicate and the error bar indicates the standard deviation.

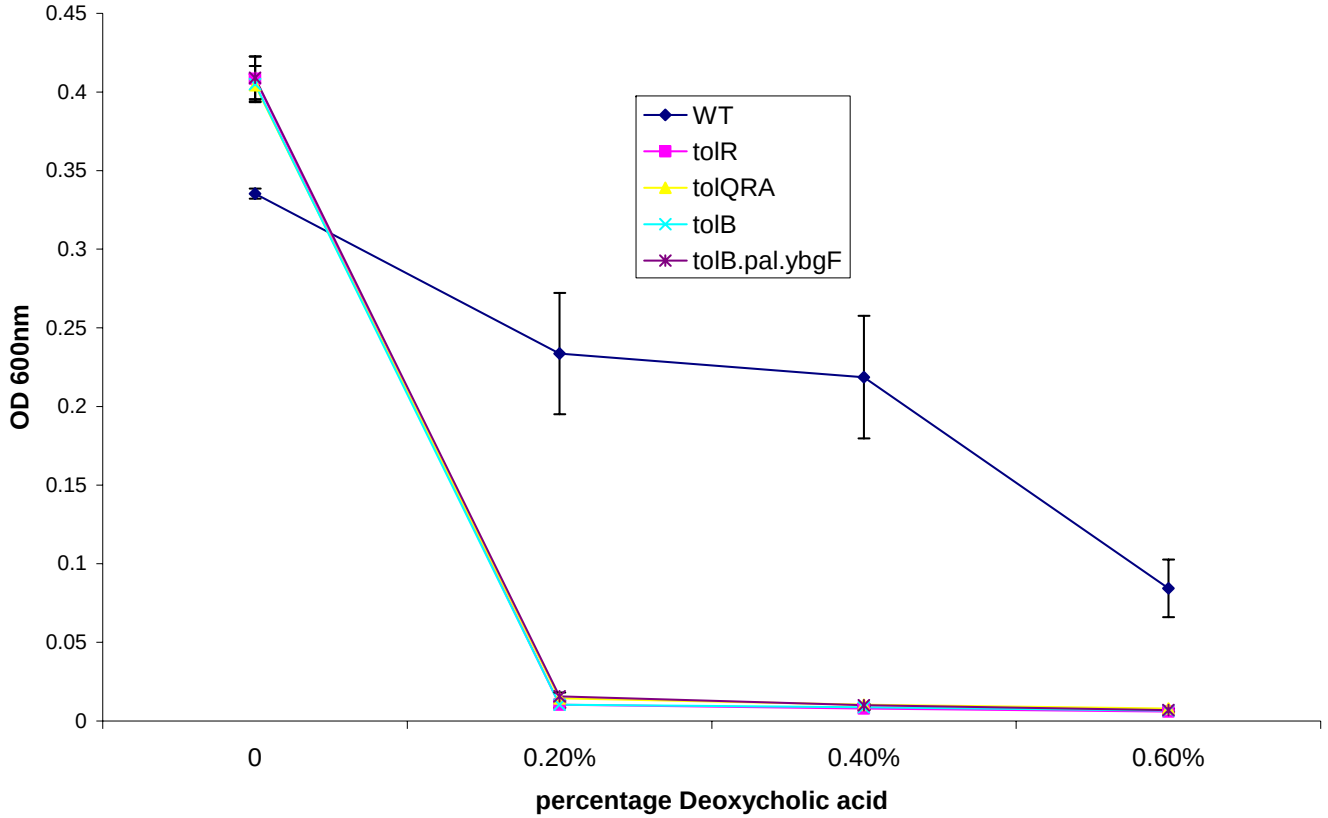


Figure 6.5 - Effect of *tol – pal* mutations on the growth of *S.* Typhimurium in liquid media supplemented with deoxycholic acid
The *tolRΔ, tolQRAΔ, tolBΔ* and *tolBpalybgFΔ* deletion mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 0.2, 0.4 and 0.6% deoxycholic acid at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

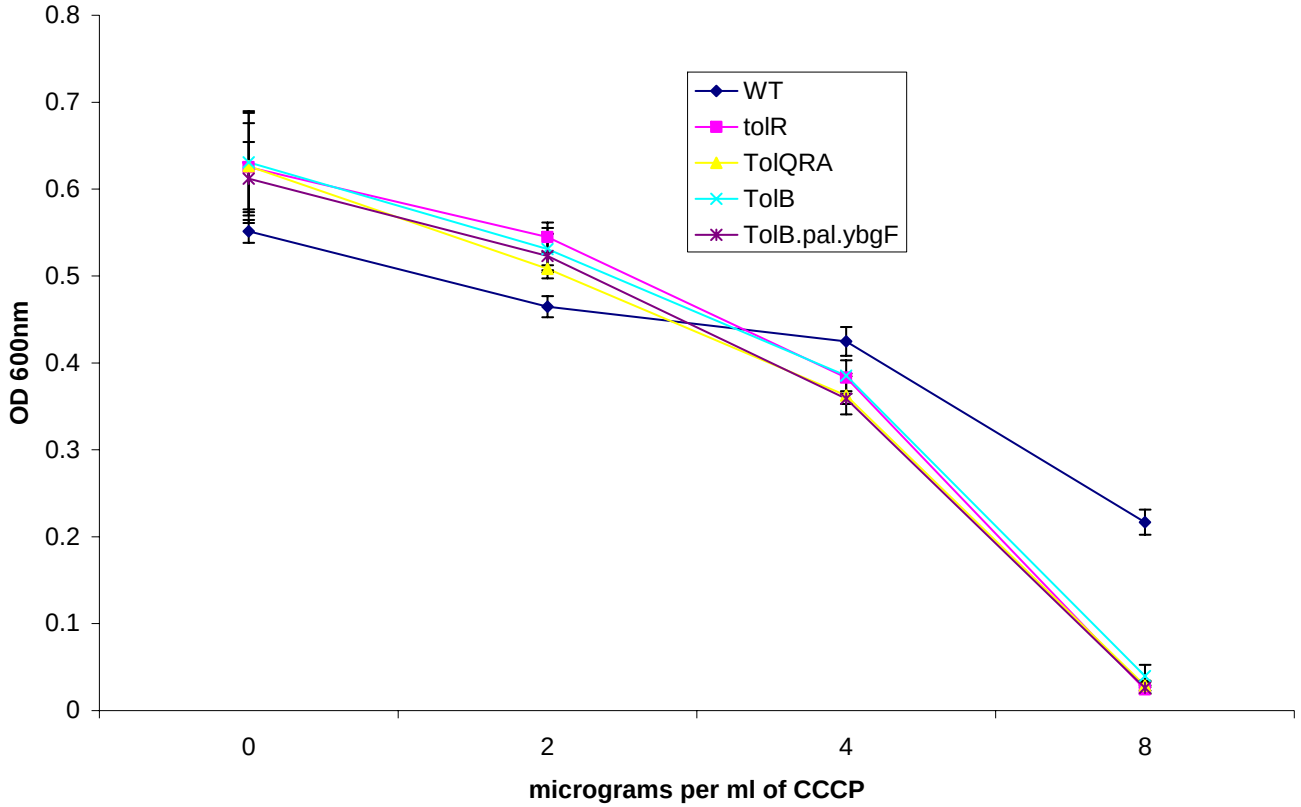


Figure 6.6 - Effect of *tol – pal* mutations on the growth of *S.* Typhimurium in liquid media supplemented with CCCP
The *tolRΔ, tolQRAΔ, tolBΔ* and *tolBpalybgFΔ* deletion mutations were compared with the WT strain for their ability to grow in culture supplemented with 0, 2, 4 and 8µg of CCCP at 37º. Growth was monitored for 16 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

6.2.5 *In vivo* analysis of *tol – pal* mutations in *S.* Typhimurium

Previous work within our laboratory concluded that a *tolRΔ* mutation in *S.* Typhimurium is extremely attenuated by both the systemic and natural route of infection. Systemic administration by IP injection resulted in a CI of ~ 0.007 which is extremely significant ($p<0.01$, student t test) when compared with wild type *S.* Typhimurium (158).

Following this, studies were established to investigate the affects of a Δ*tolR* mutant on survival after oral infection. A $6x10^6$ cfu/200µl dose was administered by oral gavage to 5 BALB/c mice and again organs were harvested five days post infection. A four fold reduction in the CFU of Δ*tolR* (5×10^1) was recovered from both spleens and livers in comparison to the wild type strain (5×10^5). In the MLN a three fold reduction in CFU of Δ*tolR* was recovered (5×10^1), in comparison with the wild type strain (8×10^3), with just a single log fold reduction seen in the Peyer's patches ($8x10^1$ versus $1x\ 10^3$). The CFU/organ for each organ investigated was significantly reduced (student t test, $p<0.05$) for the *tolR* mutant when compared with the wild type strain.

Taking the above *in vivo* studies into consideration alongside the *in vitro* results shown previously, I decided to test all four *tol – pal* mutations in a murine model by IP injection to establish if this attenuation is observed in all four strains. It was also interesting to investigate whether the effects upon virulence are more prominent in either the *tolQRA* deletion construct or the *tolBpalybgF* deletion construct. To investigate any role of virulence *in vivo* from our 4 *tol – pal* mutations, competition assays were performed as detailed in 2.8.5.1. Briefly, mice were given approximately 10^3 CFU of both WT *S.* Typhimurium and the mutant strain under investigation by IP

injection. Results showed all *S.* Typhimurium *tol* mutants to have significant attenuation when looking at both the liver and spleen in comparison to SL1344 WT in both organs. Table 15 highlights the average CI obtained for each of these mutant strains. A CI of ~1.0 indicates that the strains compete equally well. Statistical significance of the bacterial counts was measured using a two tailed unpaired t test, which showed that the degree of attenuation of *tolR, tolQRA, tolB* and *tolBpalybgF* deletion mutants is extremely significant ($p<0.01$, student t test) when compared with wild type *S.* Typhimurium.

This corresponded well with previous data obtained within our laboratory looking at the level of attenuation in a Δ*tolR* mutant in *S.* Typhimurium.

Table 15 - Effect of *tolQRA, tolBpalybgF, tolR* and *tolB* deletion mutations on *S.* Typhimurium virulence. Groups of 5 mice were challenged with a mixed dose of ~10^3 CFU WT SL1344 and the appropriate mutant strain via IP injection. The average competitive indices for each strain are shown below.

Strain	Spleen C. I	Liver C. I
tolQRAΔ	0.0000097	0.000017
tolBPalYbgFΔ	0.000044	0.000044
tolRΔ	0.000087	0.000086
tolBΔ	0.000028	0.000046

6.2.6 Tol – Pal vaccine studies

Results from the above IP studies proved that all four deletion mutants of the Tol – Pal pathway showed extremely significant levels of attenuation, which prompted us to establish further studies to investigate the potential of these genes as possible vaccine candidates. It was decided that I would study the two entire deletion mutations

tolQRA and *tolBpalybgF* to observe if one of these operons appeared to have a more positive effect on protection against SL1344 WT than the other. To initiate these vaccine studies two groups of 8 mice were infected by the oral route of infection with approximately 3 x 10^9 CFU/ml of SL1344 *ΔtolQRA* or 2.9 x 10^9 CFU/ml SL1344 *ΔtolBpalybgF* as shown in 2.8.5.3. These mice were then observed daily for one month prior to being challenged with WT *S.* Typhimurium by the oral route at a concentration of 2 x 10^7 per mouse. At this stage a control group of 4 mice, which had not been pre-immunised were also infected with WT *S.* Typhimurium by the oral route at a concentration of 2 x 10^7 per mouse. Mice were weighed and observed daily. Figure. 6.7 shows the survival rates for each group of mice. As can be seen from this Figure, all mice within the control group, which had not been pre-immunised had died by day 6 following challenge with SL1344 WT. Both groups of mice pre-immunised with either Δ*tolQRA* or Δ*tolBpalybgF* survived the SL1344 WT challenge much better. From the group of 8 mice pre-immunised with *ΔtolQRA* , 5 mice remained alive and well 19 days after WT challenge, whilst 6 mice remained alive and well out of the initial group of 8 mice pre-immunised with *ΔtolBpalybgF* after 19 days. At this stage, the experiment was stopped as it appeared that the remaining mice had survived well and were not deteriorating in condition. Study of the weight of each mouse is shown in Figure. 6.8 and it can be seen that the weight of an individual mouse appears to drop immediately before the mouse starts showing clinical signs of infection at a level upon which it must be euthanized. This can be seen most obviously in the control group of mice, which appear to lose weight immediately following challenge with SL1344 WT. In contrast, the weight of mice in the groups pre-immunised with either *ΔtolQRA* or *ΔtolBpalybgF* appears relatively constant in mice that are coping well, and it is only in the few mice in these groups that subsequently showed clinical

signs of infection and were euthanized, that showed a drop in body weight. As such, daily weighing of mice could be taken as a good indicator of health and progress of mice under such studies.

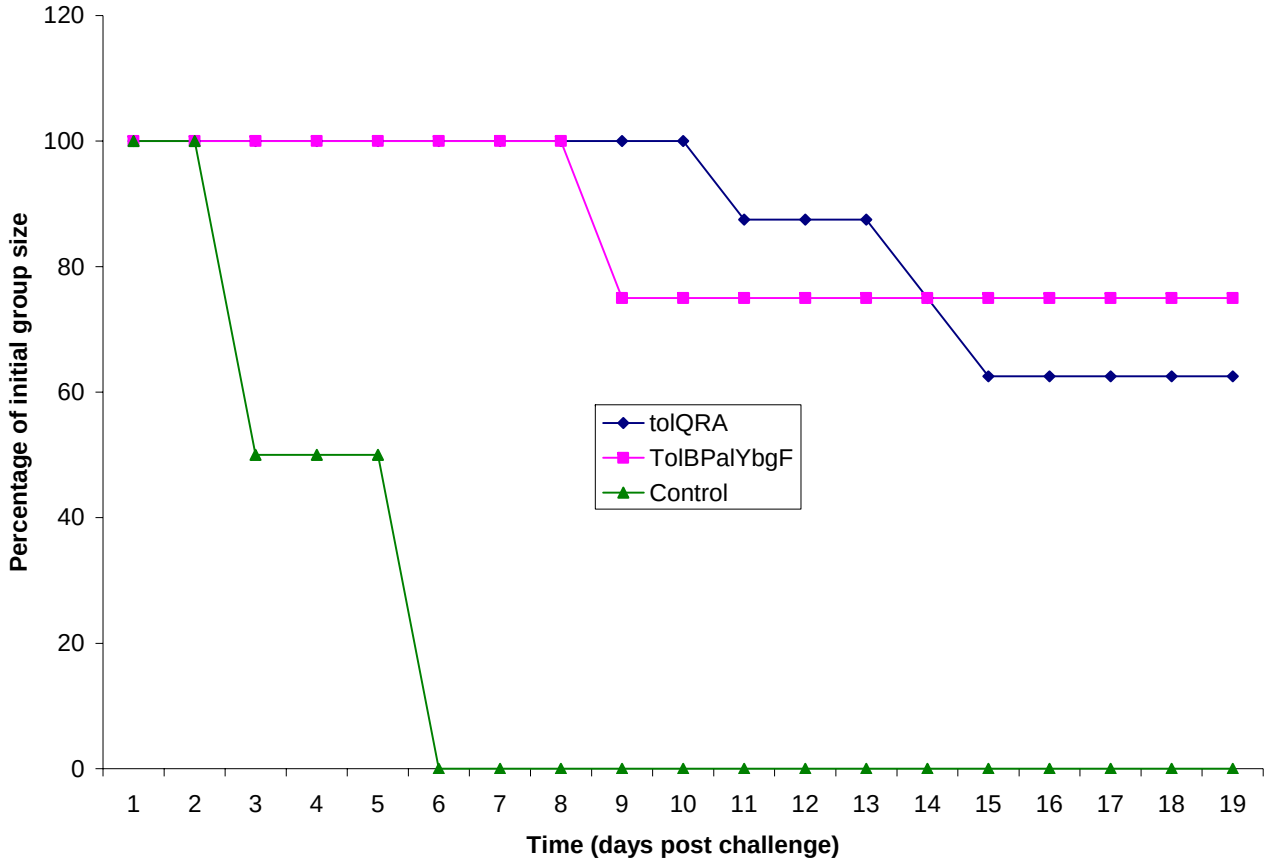


Figure 6.7 – Immunisation study studying survival of mice immunised with Δ*tolQRA*, Δ*tolBpalybgF* or a naïve control. Groups of mice (number = 8) were pre-immunised with either *ΔtolQRA* or *ΔtolBpalybgF* and challenged with SL1344 WT one month later. Control group of mice (number = 4) were also challenged with SL1344 WT but had not been pre-immunised. Survival is shown in days following SL1344 WT challenge as a percentage of the initial group size.

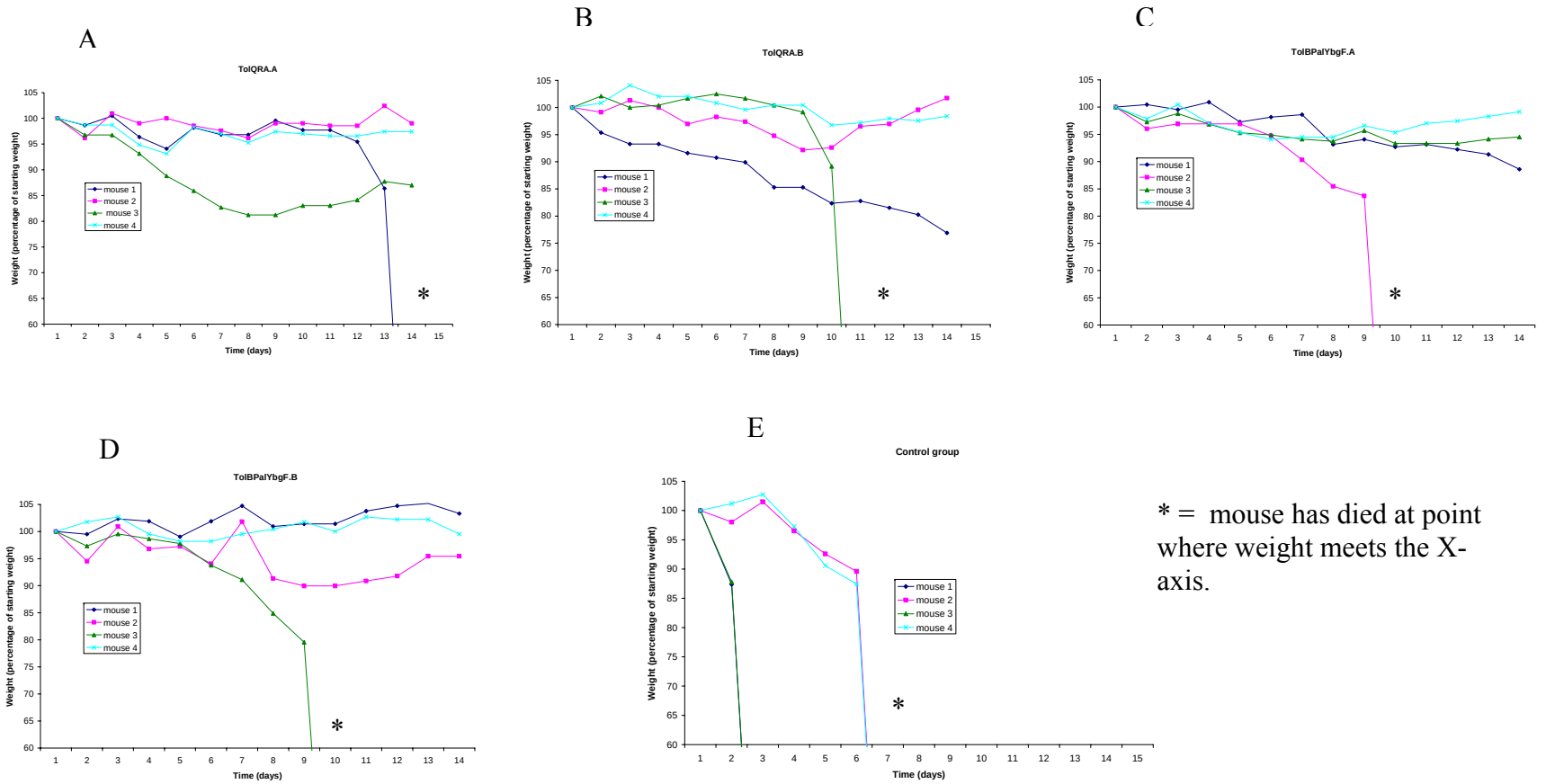


Figure 6.8 – Immunisation study studying weight of mice immunised with *ΔtolQRA*, *ΔtolBpalybgF* or a naïve control. Groups of mice (number = 8) were pre-immunised with either *ΔtolQRA* or *ΔtolBpalybgF* and challenged with SL1344 WT one month later. Control group of mice (number = 4) were also challenged with SL1344 WT but had not been pre-immunised. Mice were weighed daily following challenge with SL1344 WT and weight is shown as a percentage of the initial starting weight of each mouse. Groups A and B were pre-immunised with *ΔtolQRA*Δ, groups C and D were pre-immunised with *ΔtolBpalybgF*, and group E were the control group of mice.

6.3 Discussion

This chapter discusses the overlap between two of the extractyoplasmic stress responses, CpxAR and RpoE. Several genes co-regulated by these two systems have already been identified including *htrA* and *skp(66, 78)*.

*S.*Typhimurium HtrA is a periplasmic protease and chaperone which plays a major role during systemic *Salmonella* infection of mice (49, 167). Skp is a periplasmic chaperone important for the folding of outer membrane proteins (52). Similar to *htrA*, a S. Typhimurium *skp* mutant has been found to be highly attenuated in mice (Mark Roberts, personal communication).

As *S.* Typhimurium *htrA* and *skp* are highly attenuated we sought to investigate further genes that are co-regulated by both the RpoE and CpxAR systems in the hope of identifying vaccine candidate genes.

The *tol-pal* genes are a set of genes identified for further study on the grounds that they are likely to be regulated by both RpoE and CpxAR, and one of these genes, *tolR*, was previously shown in our laboratory to play an important role in the pathogenesis of *S.* Typhimurium (331). Both *tolR* (contained within the *tolQRA* operon) and *pal* (contained with the *tolBpalybgF* operon) were up-regulated in a *S.* Typhimurium microarray when RpoE was over expressed and also when the Cpx system was activated (as shown in table 14). Also, a *S.* Typhimurium RpoE dependent promoter controlling *tolR* has been located in the coding region of the *tolQ* gene (349). To investigate further whether any of these *tol – pal* genes are regulated by the CpxAR system RT-PCR was performed using RNA prepared from strains SL1344 CpxA* and SL1344 ΔCpxA. Expression of genes *tolQ* and *tolB* was analysed as these two genes are the first genes of the two *tol – pal* operons and results showed slight up-regulation for both genes in the induced SL1344 CpxA* strain vs. the SL1344 ΔCpxA

strain corresponding with what is seen in our microarray experiments. This experiment needs repeating for further verification, and similar RT-PCR data is also required to study the expression of all the *tol – pal* genes under RpoE inducing conditions to further support the microarray data and verify my hypothesis that these genes are both RpoE and Cpx regulated.

Following on from the RT-PCR analysis, strains were constructed in which the following genes were deleted : *tolQRA*, *tolBpalybgF* and *tolB*. Alongside our existing *tolR* mutant, these strains were tested under various conditions and showed several obvious phenotypes.

All four mutants were more sensitive to SDS compared than the WT strain, with the *tolR* and *tolQRA* deletion mutants having the greatest defect. Sodium cholate proved toxic to all 4 mutants, yet not to the WT strain and similarly these mutants exhibited increased inhibition compared to WT in the presence of deoxycholic acid. These results are not surprising as previous data has shown the *tolQRA* cluster of *S.* Typhimurium to be important for resistance to bile compounds (306). Similarly, *E. coli* and *Erwinia chrysanthemi tol- pal* mutants are hypersensitive to bile agents (86, 205). Mutations in the *E. coli tol- pal* genes result in a mucoid phenotype and mutation of the *E. chrysanthemi tol- pal* genes affects cell morphology. No obvious change in cell morphology was apparent in any of our four *S.* Typhimurium *tol – pal* mutants using basic microscope analysis of our strains grown on agar plates however future studies could elaborate on this further with more extensive investigation of any morphological changes when strains are cultured under different conditions.

Final phenotypic assays looked at the effect of the protonophore CCCP on the four *tol – pal* deletion mutants. CCCP greatly inhibited the growth of all four mutants compared to WT *S.* Typhimurium. In summary, our *in vitro* experiments showed that

all four *tol* mutants displayed statistically significantly increased levels of sensitivity to SDS, sodium cholate, deoxycholic acid and CCCP compared to WT *S.* Typhimurium. CCCP was shown earlier in my thesis to affect an *S.* Typhimurium *smpA* mutant and research by others showed it inhibited a *S.* Typhimurium *rpoE pspA* double mutant (17) indicating it may be a useful compound for testing future ESR mutants.

I.P infection studies showed that all four *tol – pal* deletion mutants are highly attenuated illustrating that the *tol-pal* genes play a role in the virulence of S. Typhimurium. This expands on previous research showing a mutation in S. Typhimurium *tolB* to affect virulence (366).

One of the main aims within this thesis was to identify any genes regulated by either the RpoE or CpxAR ESR systems which could have possible vaccine potential. With such a high degree of attenuation observed in these strains it was felt that these *tol – pal* mutants could be the ideal candidate vaccine strains. As such I investigated the utility of *S.* Typhimurium *ΔtolQRA* and *ΔtolBpalybgF* mutants as live oral vaccine candidates. Mice were immunised with SL1344 *ΔtolQRA* or *ΔtolBpalybgF* and then one month later they were challenged with WT *S.* Typhimurium by the oral route at a dose several logs higher than the LD50 (167). Results from these studies showed that mice orally immunised with either *S.* Typhimurium *ΔtolQRA* or *S.* Typhimurium *ΔtolBpalybgF* were much more likely to survive a challenge with WT *S.* Typhimurium compared to the control group. The control group of mice deteriorated much quicker illustrating the virulence of the challenge strains. The group size in this experiment is relatively small and future studies using greater numbers may give more statistical significance to the data. Also, the immunisation dose could be increased to see if this has a more positive effect on the protection of mice. It may be that

mutations in the *tol-pal* genes are over-attenuated causing a reduction in virulence too great to be of use as a candidate vaccine strain as is seen for a *S.* Typhimurium *rpoE* mutant (167). If this is the case we can examine other genes co-regulated by the RpoE and CpxAR ESR systems for their potential as live vaccine candidate genes. For example the *manX, Y* and *Z* genes for example are up-regulated in both the RpoE and CpxAR arrays. This group of genes is known to affect the surface motility of S. Typhimurium and it would be interesting to investigate this further (386).

To expand on my present studies of the *tol-pal* genes it would be interesting to examine the effect of these genes in *S.* Typhimurium on the surface expression of LPS as studies have shown the *tol-pal* genes to affect 07 and 016 antigen LPS expression in *E. coli* (380).

Chapter Seven – Study of HtrA in *S.* Typhimurium

7.1 Introduction

HtrA is a stress response protein that is essential for *Salmonella* species to survive within macrophages and cause systemic infection.

An interesting characteristic of HtrA is that its activity is ATP independent unlike other heat shock proteins (363) and it is one of a family of proteins that can function as both a protease and a chaperone (279). It is currently unknown which of the biological activities of HtrA, enzymatic or chaperone, is important for *Salmonella* during infection and this will be investigated in this chapter.

In addition to HtrA, *S.* Typhimurium also possesses two paralogues called DegQ (HhoA) and DegS. DegQ is not essential for *S.* Typhimurium pathogenesis, previous studies within our laboratory have shown a *degQ* mutant to be as virulent as WT *Salmonella* in a mouse model, although a *degQ htrA* double mutant was shown to survive less well in mouse organs than a *htrA* mutant alone (101). DegS however is similar to HtrA in that it is important for survival at elevated temperatures and is necessary for full virulence in a mouse model (260, 333).

HtrA, DegS and DegQ have similar structural domains with a catalytic domain containing a catalytic triad of serine proteases, and C terminal PDZ domains. PDZ domains are protein modules that mediate specific protein-protein interactions and bind preferentially to the C-terminal three to four residues of the target protein (54). The only structural difference between HtrA and DegQ, is the absence of a flexible Q-linker in the N-terminus of DeqQ. HtrA and DegQ have two PDZ domains while DegS has only one PDZ domain, and further differences between these paralogues are found at the N-terminus. A further difference is that the proteolytic activity of DegS

requires activation by an extracellular signal, whereas DegQ and HtrA are proteolytically constitutively active (198, 385, 397).

The importance of the enzymatic and chaperone activities of HtrA to its virulence status in *Salmonella* is presently unknown. The PDZ domains operate to mediate protein to protein interaction and are thought to be very important to the action of HtrA. As such in this chapter I investigate the individual components of *S.* Typhimurium HtrA with the development of a variety of deletion constructs lacking different structural elements of HtrA to allow investigation of its proteolytic and chaperone activity as well as the role of the PDZ domains. Figure. 7.1 shows the structural domains of *S.* Typhimurium HtrA.

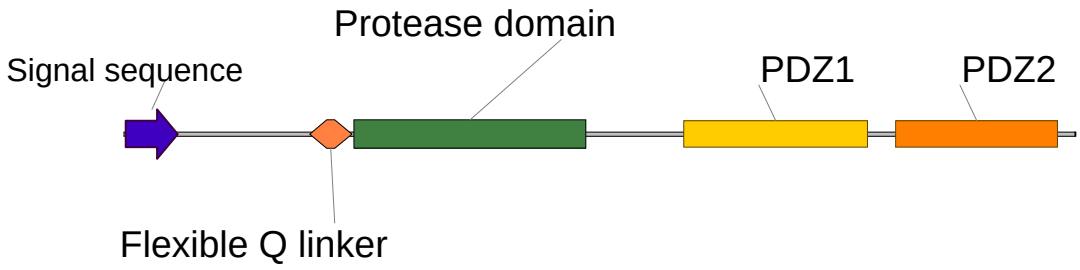


Figure 7.1 – Structural domains of *S.* Typhimurium HtrA.

A study investigating the proteolytic and catalytic activity of HtrA in *E.coli* was performed which involved the construction of six *htrA* deletion constructs (355). One construct contained an entire fully functioning *htrA*, one was a proteolytically inactive variant of *htrA*, one had a deletion of the protease, one lacked PDZ domain 1, one lacked PDZ domain 2 and the final construct lacked both PDZ domains. Complementation studies were established to investigate the ability of these

constructs to restore the temperature sensitive (T^s) phenotype of *E. coli htrA*. Results from these studies showed that the T^s phenotype could only be restored fully in the presence of the entire fully functioning construct. A small degree of complementation was observed in the proteolytically inactive *htrA* construct and no complementation was detected in all other constructs lacking either the protease or any of the PDZ domains.

I obtained the above *E. coli htrA* constructs for use in our study to assess which regions of *htrA* may be important in the virulence of *S.* Typhimurium.

All constructs were fused to the *htrA* promoter of *S.* Typhimurium and analysed both *in vivo* and *in vitro* to investigate which structural domains of *S.* Typhimurium HtrA are important in its pathogenesis. It is hoped this data may help in future vaccine development.

7.2 Construction of *S.* Typhimurium *htrA* deletion mutants

To allow us to investigate the properties of the *E. coli htrA* constructs in *Salmonella* we first had to fuse these constructs to the *htrA* promoter of *S.* Typhimurium by a process called sewing PCR.

The 5 htrA constructs used are shown below in Figure. 7.2 and were named htrA 1 to 5 respectively. All five constructs were then transduced into an SL1344 htrAΔ background strain (GVB1343, shown in table 2) for subsequent analysis both *in vitro* and *in vivo*.

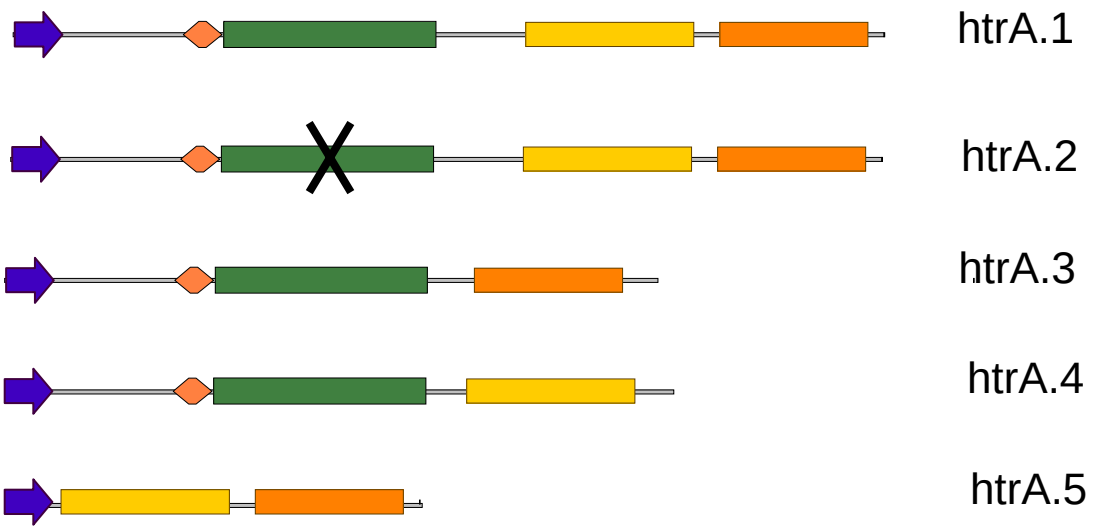


Figure 7.2 – Structural domains of htrA deletion constructs. htrA.1 contains the entire active htrA, htrA.2 has a proteolytically inactive htrA, htrA.3 lacks PDZ domain 1, htrA.4 lacks PDZ domain 2 and htrA.5 lacks both the flexible Q-linker and protease.

Recombinant DNA techniques used in the construction of the htrA variants are detailed in sections 2.3 and 2.4. Briefly, the *htrA* promoter of SL1344 *S.* Typhimurium was amplified using oligonucleotides htrAFWproCL and htrAREVproCL shown in table 4. The variant sections of *htrA* gene from plasmids pCS20, pCS21, pIW7, pIW5 and pIC1 (table 3) were amplified using oligonucleotides htrAFWCL and htrArv1bamhi. Following amplification of the *htrA* promoter and the variant *htrA* genes, a sewing PCR was set up to stitch the promoter region to the variant *htrA* gene regions. As the oligonucleotide htrAREVproCL contained homology to the tail of the variant *htrA* genes, a PCR was set up using oligonucleotides htrAREVproCL and htrArv1bamh1 with a 1:1 ratio of *htrA* promoter product to modified *htrA* gene product as the DNA source. The resultant amplified products produced 5 constructs comprising the *htrA* promoter fused to pCS20, pCS21, pIW7, pIW5 or pIC1. These constructs were subsequently renamed htrA.1, htrA.2,

htrA.3, htrA.4 and htrA.5 respectively. Oligonucleotide htrAFWCL was designed to incorporate the restriction site HindIII and oligonucleotide htrArv1bamhi incorporated the BamH1 restriction site allowing the 5 new *htrA* constructs to be cloned into the MCS of the low copy number plasmid pWSK29. All *htrA* constructs (shown in table 16) were then electroporated into SL1344 *htrA*Δ.

Table 16 – A List of htrA constructs used in this study.

Construct	Genotype
GVB1343 phtrA1	SL1344 ΔhtrA, htrA1 in pWSK29
GVB1343 phtrA2	SL1344 ΔhtrA, htrA2 in pWSK29
GVB1343 phtrA3	SL1344 ΔhtrA, htrA3 in pWSK29
GVB1343 phtrA4	SL1344 ΔhtrA, htrA4 in pWSK29
GVB1343 phtrA5	SL1344 ΔhtrA, htrA5 in pWSK29

PCR amplification was performed using oligonucleotides htrAFWproCL and htrARv1bamhi to confirm that each structural variant and promoter fusion was the expected size (Figure. 7.3).

Western blotting using a anti-HtrA antibody was also used to confirm expression of htrA variants htrA.1, htrA.2, htrA.3, htrA.4 and htrA.5 respectively.

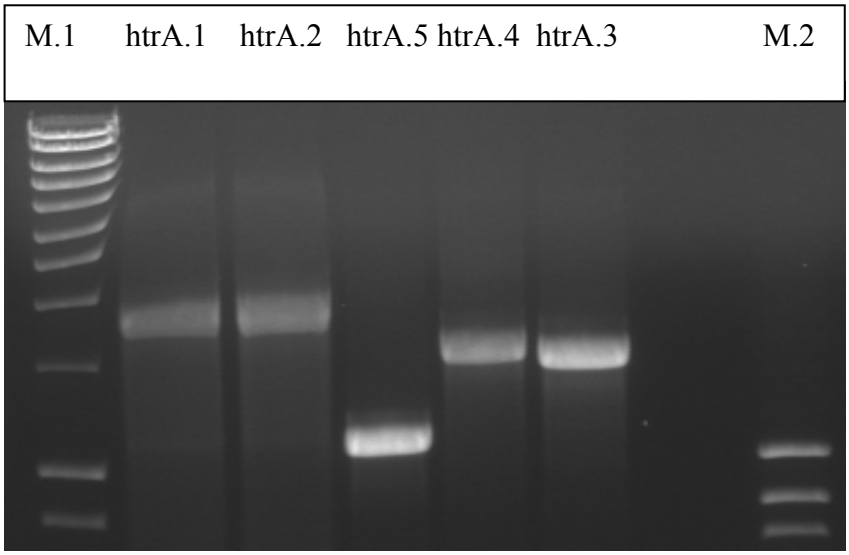


Figure. 7.3 – PCR verification of variant *htrA* constructs. PCR verification was performed as in 2.7.2, using oligonucleotides htrAFWproCL and htrARv1bamhi. All products were sized as determined by Hyperladder I DNA ladder (M.1) and Hyperladder IV DNA ladder (M.2).

7.3 *In vitro* analysis of *S.* Typhimurium *htrA* constructs

Following construction of the modified *htrA* strains, growth was monitored at 46ºC, as this is the temperature at which there is an observable difference between WT SL1344 *S.* Typhimurium and GVB1343 (SL1344ΔhtrA). Results from these experiments (Figure. 7.4) show that GVB1343 phtrA1 and GVB1343 phtrA2 perform similarly to WT *Salmonella*, with htrA.1 showing a slight increase compared to htrA.2. This is as expected as htrA.1 contains the entire non-disrupted *htrA*, whilst htrA.2 is the proteolytic inactive variant of *htrA*.

As shown previously GVB1343 shows much decreased growth at 46ºC compared to WT and results here show that the GVB1343 phtrA.3, GVB1343 phtrA.4 and

GVB1343 phtrA.5 modified *htrA* constructs show an even greater degree of decreased growth.

7.4 *In vivo* analysis of *S.* Typhimurium *htrA* constructs

As mentioned previously, GVB1343 shows reduced growth in mouse tissue following infection, though it is still able to colonise both the liver and spleen of the animal. To investigate the effect of each modification to the *htrA* gene, all 5 strains were used in a mouse model infection study. All animals were challenged intraperitoneally with 5 x 10^3 bacteria and culled after 3 days, following which bacterial counts on the liver and spleens of all animals was performed. Results from these experiments showed that the GVB1343 phtrA.1 had approx 1.7 CFU / organ in both the liver and the spleen, as a proportion of the initial inoculum following bacterial counts. GVB1343 phtrA.2 had approx. 1.2 CFU / organ, GVB1343 phtrA.4 and GVB1343 phtrA.5 had approx. 0.9 CFU/ organ, whilst GVB1343 htrA.3 showed the least colonisation with approx. 0.8 CFU / organ (Figure.7.5). The data from these *in vivo* experiments correlates well with the *in vitro* data shown above, where by GVB1343 phtrA.1, containing the entire non disrupted *htrA*, shows the greatest effect. This is closely followed by htrA.2, containing the entire proteolytically in-active HtrA. The other three HtrA variants all show much reduced degrees of infectivity. Using ANOVA to statistically compare the degree of infectivity, both GVB1343 phtrA.1 and GVB1343 phtrA.2 were found to be significantly different compared to the empty vector control, with a p value in both cases of <0.001. There was found to be no significant difference between the empty vector control and GVB1343 phtrA.3, GVB1343 phtrA.4 and GVB1343 phtrA.5, all having P values > 0.005.

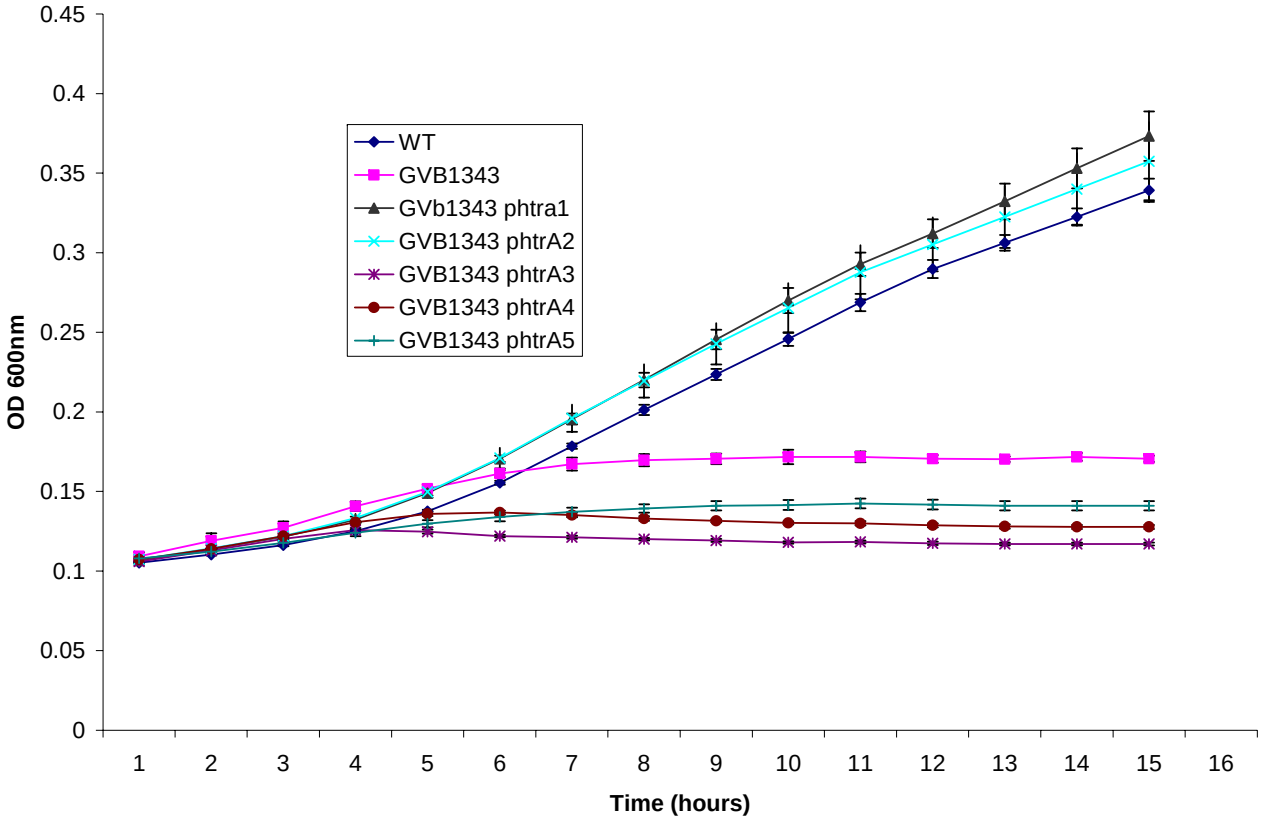


Figure. 7.4 - Effect of *htrA* mutations on the growth of *S.* Typhimurium in liquid media at 46°C
The *htrA* variant mutations were compared with the WT strain and the *htrA* full mutant for their ability to grow in culture at 46ºC using a Bioscreen machine. Growth was monitored for 8 hours and measured spectrophotometrically at OD600nm. Experiments were performed in triplicate and the error bar indicates the standard deviation.

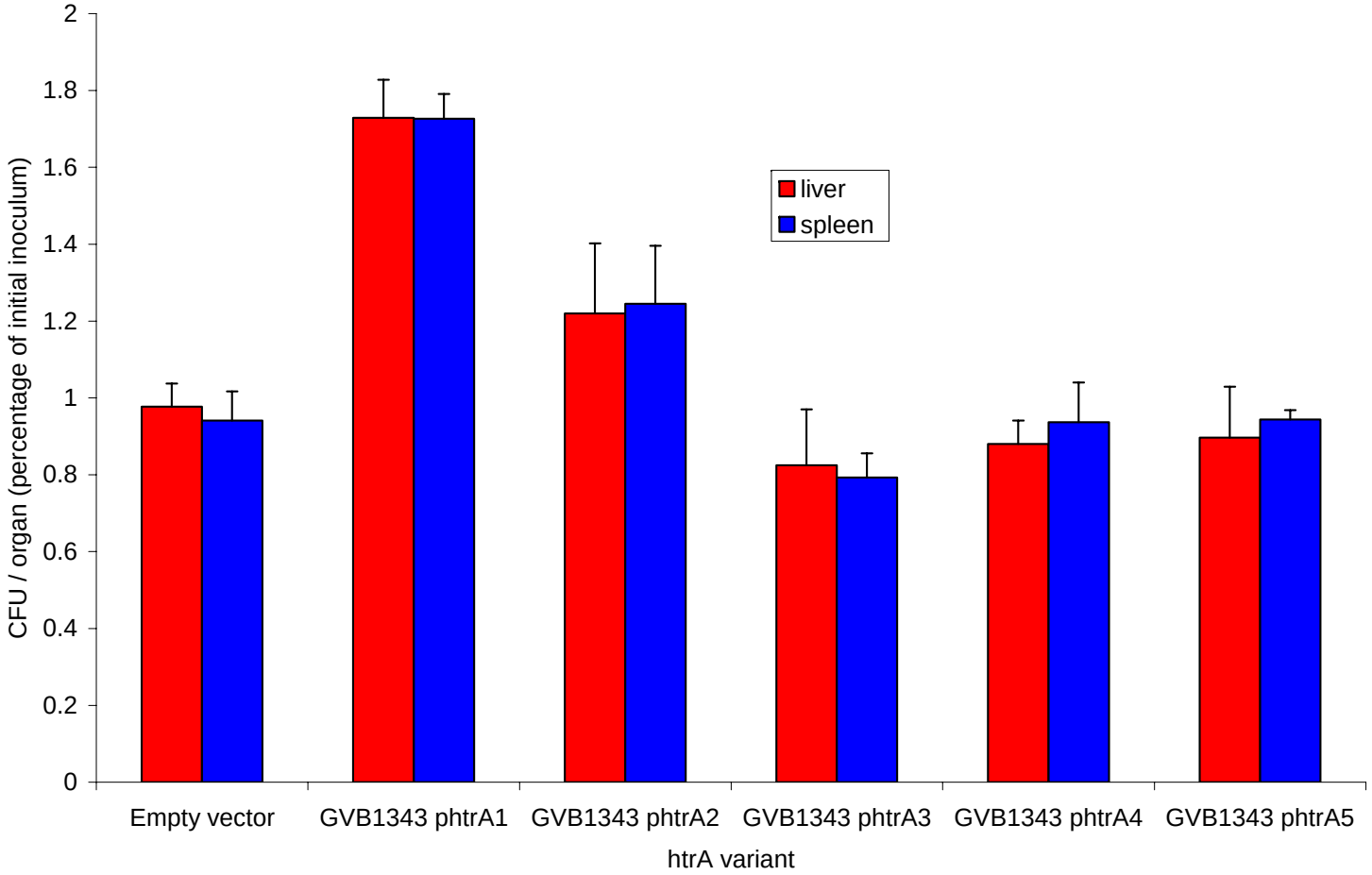


Figure. 7.5 - Effect of *htrA* mutations on the growth of *S.* Typhimurium *in vivo*. Groups of 5 mice were used and standard deviation between the mean is shown in error bars above.

7.5 Discussion

HtrA is a stress protein essential for systemic virulence of *S.* Typhimurium and the ability of the organism to survival within macrophages. *S.* Typhimurium *htrA* mutants are highly attenuated and function as effective live vaccines in mice (49). HtrA has both proteolytic and chaperone activities however it is unknown which of these are important during systemic infection. To investigate which features of HtrA are important for salmonella during systemic infection, several *htrA* constructs that encode HtrA that lack one or more domains or functions were obtained. As they were constructs they would not express in *S.* Typhimurium during infection because the promoter would not be active because of the repressor ($LacI^{Q}$) encoded by the plasmid. To produce plasmids encoding the variants that would express in *S.* Typhimurium *in vivo* PCR was used to fuse the promoter region of *S.* Typhimurium *htrA* to the *htrA* genes encoding the HtrA variants (Fig.7.2). phtrA.1 encodes WT HtrA, phtrA.2 encodes HtrA that is proteolytically inactive, phtrA.3 encodes HtrA that lacks PDZ domain 1, phtrA.4 encodes HtrA that lacks PDZ domain 2 and phrA.5 encodes HtrA that lacks both the protease domain and flexible Q linker. The new htrA plasmids were introduced into the *S.* Typhimurium *htrA* mutant GVB1343.

The GVB1343 strains expressing WT HtrA or protease negative HtrA (GVB1343 phtrA.1 and GVB1343 phtrA.2) grew as well as the *S.* Typhimurium WT strain at $46^{o}C$. This indicates that for growth at high temperature the chaperone function of HtrA alone is sufficient. This is consistent with what was found with *E.coli* that a protease minus HtrA variant complements the inability of *E.coli htrA* to grow at 42ºC, although in this case the protease minus *htrA* variant was not as effective as the WT HtrA (355). In contrast, the other *htrA* variant constructs (GVB1343 phtrA.3,

GVB1343 phtrA.4 and GVB1343 phtrA.5) did not restore the growth of GVB1343 to WT levels. Therefore, despite the fact that purified HtrA variants that are missing one or more PDZ domains can facilitate the refolding of a model substrate *in vitro* at low temperatures (355) this activity can not complement the temperature sensitivity of *S.* Typhimurium *htrA* mutants. It is possible that at high temperature the HtrA variants lacking a PDZ domain are unstable or do not fold correctly.

In vivo experiments were then carried out to investigate what role each structural component of HtrA has during *S.* Typhimurium systemic infection of mice.

The results of the *in vivo* study were similar to what was seen in the growth at high temperature studies. That is the strains expressing the protease-minus HtrA or the WT HtrA, GVB1343 phtrA.2 and GVB1343 phtrA.1, grew significantly better in the liver and spleen of infected mice than the control strain (GVB1343 with empty plasmid). However, in contrast in the *in vivo* situation the strain expressing WT HtrA grew to significantly higher levels than the strain expressing the protease negative HtrA. On the other hand, the strains expressing any of the three other HtrA variants (GVB1343 phtrA.3, GVB1343 phtrA.4 and GVB1343 phtrA.5) could not infect mice any better than the control strain with the empty vector. This indicates that both the chaperone and protease activity of HtrA are important in the systemic infection of host tissue by *S.* Typhimurium but that the protease activity is probably most important. This is an interesting result, as this has not been seen previously. In *E. coli* removal of one PDZ domain from HtrA reduces but does not abolish chaperone activity (355). However, our results show that *in vivo* as at high temperature the PDZ domains are indispensible for HtrA activity.

The importance of HtrA for *Salmonella* virulence is also surprising as *Salmonella* have another, highly homologous periplasmic protease, DegQ, that could compensate for the loss of HtrA. The main difference between DegQ and HtrA is the absence of the flexible Q linker region in DegQ. This flexible region is situated just upstream of the protease domain in HtrA. Another difference between HtrA and DegQ is in the regulation of their expression, *degQ* is constitutively expressed while *htrA* is co-regulated by both the RpoE and CpxAR stress response systems(56, 63, 65) and induced during stress conditions such as high temperature or growth within macrophages(95, 282, 284, 383). To determine if the Q linker determines the differential importance of HtrA and DegQ to virulence given more time it would be useful to mutate *htrA* to produce an in-frame deletion that removes the Q linker region and conversely add the HtrA Q linker to DegQ. These constructs could be cloned into a low copy plasmid vector and introduced into the *S.* Typhimurium htrA strain to reveal if the Q linker alone determines the biological differences between HtrA and DegQ.

It is possible that removal or addition of the Q linker molecules may affect folding and oligomerizarion of HtrA and DegQ. To study this further we could make forms of HtrA-Q linker (HtrA-Q) and DegQ-Q linker (DegQ-Q) suitable for purification. Purified HtrA-Q and DegQ-Q and WT HtrA and DegQ could then be characterised *in vitro* by looking at protease activity and oligomerisation.

It would also be useful to study the difference in regulation between *htrA* and *degQ*. With our collaborator Jan Kormanec(Slovakia) we have mapped the S. Typhimurium *htrA* promoter and showed that the promoter region of *S.* Typhimurium is larger than that of *E. coli* and also contains additional promoters (unpublished). In contrast *degQ*

is thought to be constitutively expressed from a single promoter, ie is controlled by the housekeeping sigma factor. *E.coli degQ* can complement the temperature sensitive phenotype of a *E.coli htrA* mutant (383). *htrA* is highly upregulated in S. Typhimurium growing within macrophages whereas *degQ* is not (95). It could be that HtrA is critical to *Salmonella* virulence and growth within macrophages because its production can be increased to respond to stress. To test this hypothesis we could fuse the *S*. Typhimurium *htrA* promoter to *degQ*, clone the construct into pWSK29 and study the ability to substitute for *htrA* during infection and growth at high temperature.

Chapter Eight – General Discussion

8.1 General Discussion

Although *Salmonella* is a much studied bacterial pathogen, it still causes extensive morbidity and mortality worldwide. Consequently, much research still needs to be done in the development of both new antibacterial agents and also more efficacious vaccines to treat typhoid fever.

Salmonella species encounter a wide variety of stresses in the environment and during host infection and to counter the stresses placed upon it, *Salmonella* has a number of stress response systems in place which can initiate the transcription of the relevant genes necessary under a challenging environment.

The extracytoplasmic stress response systems (ESR) are one such set of response mechanisms employed by *Salmonella* and are studied in detail throughout this thesis.

Principally, the main objective of this study was to further clarify the ESR systems of *S.* Typhimurium, beyond what is currently known, with a view to identifying genes which could hopefully be utilised as potential vaccine targets.

In chapter 3 I studied in detail 9 genes which I take to be rpoE regulated expanding our current knowledge of rpoE regulon members in S. Typhimurium. One of these genes, *smpA* (recently identified as a non-essential component of the YaeT complex) showed strong *rpoE* related phenotypes. My study of *smpA* indicates that it plays a crucial role in the pathogenesis of *S.* Typhimurium which has not been previously shown and hopefully future studies can be established to examine the vaccine potential of strains lacking this small membrane protein.

In chapter 4 phenotypic and *in vivo* study of the PSP response in *S.* Typhimurium confirmed that the *psp* operon appears to respond to membrane associated stress in a similar manner to that seen in *E. coli* (393).

I undertook to study the regulation of the psp in *S.* Typhimurium to help clarify whether this response is indeed a fourth ESR that can be regulated independently of the *rpoE* system as it is seen in *E.coli* that *rpoE* is not required for *psp* induction. Although expression of *psp* genes in *S.* Typhimurium requires a lot more work, initial studies indicate that these genes comprise an extracytoplasmic response system that can be affected by other regulatory pathways (including RpoE and RpoN) but equally its expression can be regulated independently by these pathways.

Following on from studies on the PSP response system , I began to study the CpxAR two component ESR system. A microarray was designed identifying many genes as being putatively up- or down-regulated by the CpxAR system including many which were previously known including *htrA, dsbA* and *ppiA*. This array has greatly expanded our knowledge of CpxAR regulon members in *S.* Typhimurium. In particular the identification of fimbrial associated genes and SPI-1 associated genes as CpxAR regulated was investigated further and confirmed using both RT-PCR and western blot analysis.

The discovery from my microarray studies that many SPI-1 associated genes are putatively CpxAR regulated ties in with previous evidence of a connection between these genes and the CpxAR system (92, 257) and our findings that the SPI-1 associated genes are likely to be Cpx regulated heralds an important step forward in the understanding of the Cpx response in *S.* Typhimurium. The SPI-1 genes encode a TTSS involved in the delivery of effector proteins required for invasion. It also known that this system is implicated in the development of the enteritis infection and their

role in the Cpx system could explain why the Cpx system itself appears to be more implicated in the initial stages of infection rather than the later stages of infection.

To investigate the co-regulation of genes by both the Cpx and RpoE ESR systems, I studied a group of genes called *tol – pal* that appeared to be regulated by both these systems as I hypothesise that genes regulated by several different systems will play an important role in the bacteria

I have shown the *tol – pal* genes of S. Typhimurium to be strongly affected by membrane affecting compounds and also very attenuated in a mouse model of infection. These novel studies show the *tol-pal* operon to be crucially importrant in the ability of *S*. Typhimium to respond to environmental stresses (such as CCCP exposure) and also stresses present within the host (including bile acid resistance). Given the high level of attenuation observed for our *tol-pal* genes, these genes were selected as candidate vaccine genes in preliminary vaccine studies with promising results. Our analysis of all the data obtained on the *tol – pal* genes indicates that they are co-regulated by both the *rpoE* and Cpx ESR systems and very important to the virulence of *S*. Typhimurium. It is anticipated that our initial immunisation studies can be taken further to investigate the potential of these genes as true vaccine candidates. Their utility as vaccine candidates highlights the importance of study into genes co-regulated by several different systems.

The final results chapter within this thesis concentrated on study of *htrA* in *S*. Typhimurium. As mentioned previously, this is another gene known to be co-regulated by both the *rpoE* and Cpx ESR systems that is also very important to the virulence of *S*. Typhimurium. Results showed that the constructs which lacked either PDZ domains 1 and 2, PDZ domain 1 or the protease and flexible Q-linker could not complement the temperature sensitive phenotype seen in *htrA*, and actually these three

constructs exhibited an even greater decrease in growth at 46ºC than an SL1344 *htrA* mutant itself. Subsequent *in vivo* studies showed the same three constructs to be very attenuated in mice. Taken together these studies indicate that BOTH the protease and chaperone activities of *htrA* are important in the systemic infection of host tissue by *S.* Typhimurium, which has not been previously illustrated. Future studies will hopefully establish more fully the role of the flexible Q- linker in S. Typhimurium *htrA* which should also help widen our knowledge of the need for *Salmonella* to possess both *htrA* and *degQ*.

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