Characterisation of the *S.* Typhimurium σ^E Regulon

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Statement

I hereby declare that this submission is my own work and to the best of my knowledge contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the University or other Institute of higher learning, except where due acknowledgment has been made in the text. The work involving identification of RpoE regulated genes by the two plasmid system was part of a collaborative project and performed by the group of Dr Jan Kormanec, Slovakia.

Signed..... (Gary Rowley

Date...16/5/05.....

Abstract

The extracytoplasmic stress response (ESR) of the *Enterobacteriacea* consists mainly of two partially overlapping pathways, one regulated by the alternative sigma factor, σ^E, encoded for by *rpoE* and the other by the two component regulator *cpxRA*. This thesis mainly focuses on the σ^E regulated arm of the ESR but does discuss potential crosstalk between these two systems.

Characterisation of the *rpoE* regulon in *E. coli* has been performed using a number of methods, each of which identified different genes (Dartigalongue, Missiakas, and Raina, 2001;Rezuchova *et al.*, 2003). RpoE has been described as essential for survival of *E. coli* laboratory strains (De Las, Connolly, and Gross, 1997a), but deletion of the *rpoE* structural gene in *S.* Typhimurium is not lethal (Humphreys *et al.*, 1999). However the *S.* Typhimurium *rpoE* mutation is critical for survival *in vivo*, as it appears to be for a number of pathogenic bacteria (Humphreys *et al.*, 1999;Hild *et al.*, 2000;Craig, Nobbs, and High, 2002;Heungens, Cowles, and Goodrich-Blair, 2002;Kovacikova and Skorupski, 2002;Testerman *et al.*, 2002;Heusipp, Schmidt, and Miller, 2003). Considering the different locations and microenvironments encountered by these different pathogens it is likely that each of the *rpoE* regulons may contain different genes. To establish which are important for *S.* Typhimurium our collaborators and I have utilised a number of techniques to identify RpoE regulated genes. In collaboration with the group of Dr. Jan Kormanec, Slovakia, a two plasmid system has been utilised. We have also been given access to the *S.* Typhimurium microarrays at the Institute of Food Research, and used a promoter consensus search derived from known σ^E regulated genes to search the *S.* Typhimurium genome for putatively σ^E regulated genes. With a combination of these techniques we have identified a number of putatively σ^E regulated

genes, and have selected a portion of these to be mutated with lambda red mutagenesis and characterised both *in vitro* and *in vivo*.

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Abbreviations

Lpf	Long polar fimbriae
HilA	Hyperinvasion locus protein A (transcriptional activator)
SprB	Salmonella pathogenicitiy Island 1 regulator
HilC	Hyperinvasion locus protein C (transcriptional regulator)
HilD	Hyperinvasion locus protein D (transcriptional regulator)
InvF	Invasion protein F
SopE	*Salmonella* outer protein E (SPI-1 effector protein)
SopE2	*Salmonella* outer protein E 2 (SPI-1 effector protein)
Cdc42	Cell division control protein 42
Rac1	Ras related C3 botulinum toxin substrate (GTP binding protein)
p38	Intracellular stress activated Map kinase
ERK	Extracellular signal regulated kinase 1
JNK	C-jun N terminal kinase (stress activated kinase)
Bcg	Synonym for Nramp
DnaA	DNA initiation protein
H-NS	DNA binding nucleoid associated protein
CRP	cAMP receptor protein
Fis	Factor for inversion stimulation
Hfq	Host factor I protein
RseA	Regulator of sigma E activity
DegS	Degradative protease S (Serine protease).
YaeL	Systematic nomenclature (serine protease)
AlgU	Alginate Regulatory protein U
FLP	Flippase recombinase

CpxRA Conjugative plasmid expression (Two component regulator)

Pnpase Polynucleotide phosphorylase

SbmA Sensitivity to microcin B17 protein

YaiW Systematic nomenclature (function unknown).

CDS Coding sequence

FhuA Ferric hydroxamite uptake

Skp Seventeen Kilo-Dalton protein (chaperone)

FirA Synonym for LpxD

FkpA FKBP type peptidyl proly isomerase

OmpA Outer membrane protein

YbgC Systematic nomenclature (function unknown)

TolQRA Tolerance proteins QRA

TolB Tolerance protein B

Pal Peptidoglycan associated lipoprotein

YbgF Systematic nomenclature (function unknown)

MudJ Mu defective transposon element J

LpxD UDP-3-O-[3-hydroxymyristoyl]-glucosamine N-acetlytransferase

ABC ATP binding cassette superfamily

DM Double mutant

YccV Systematic nomenclature (function unknown)

Mlc Making large colonies protein

NagC N-acetyl glucosamine protein C

YggN Systematic nomenclature (function unknown)

EcfF Extracellular function protein F (Yael/RseP)

YggT Systematic nomenclature (function unknown)

RpoE RNA polymerase subunit E (alternative sigma factor)

Chapter 1 – Introduction

1.1 The Genus *Salmonella*

Salmonellae are a group of closely related bacteria with the ability to cause a variety of clinical manifestations in several diverse species, from nematodes to mammals (including humans) and birds. The genus *Salmonella* are members of the family *Enterobacteriaceae* and are fermentative, facultatively anaerobic intracellular pathogens which are oxidase negative. The salmonellae are Gram negative bacilli approximately 2-3 x 0.4-0.6 μm in size, most species of which are motile.

1.1.1 Classification

Classification of species has been based on a variety of confusing categories including host specificity, the presence of cell surface antigens such as lipopolysaccharide (LPS) and sensitivity to phage. The most recognised of these classifications is based upon surface antigens, which allowed accurate serological characterisation of salmonellae. (Edwards and Kauffmann, 1952) utilised variation of flagellar (H) antigens, and devised a scheme based on monophasic and diphasic serotypes (also known as phase 1 (H1) and phase 2 (H2). This scheme was then altered by Kauffmann over a period of years to include classification based on cell surface LPS antigen (O or somatic antigen) of which there are over 50 major types determined by the type, arrangement and condition of the oligosaccharide subunits. The Kauffmann-White schema adopts the principle that each different combination of O, H1 and H2 antigens is a separate serovar forming a complicated salmonellae classification system containing over 2400 serovars. These are normally given names after their host and disease symptoms (*Salmonella choleraesuis*) or where they were first identified (*Salmonella dublin*) (Edwards and Kauffmann, 1952;Kauffmann, 1957;Kauffmann and Edwards, 1957;Le Minor and Bockemuhl, 1988). Using biotyping and a limited amount of DNA-DNA hybridisations these

serovars were later grouped as seven subspecies (I, II, IIIa, IIIb, IV, V, and VI) (Crosa *et al.*, 1973;Stoleru, Le Minor, and Lheritier, 1976;Le Minor, Veron, and Popoff, 1982). 60% of serotypes belong to subspecies I, and includes greater than 90% of strains that cause human salmonellosis (Neidhardt, 1996).

Although important at the time, with the advent of genomic based technologies, the antigenic schema and subspecies classification is now invalid. Initially genomic sequencing focused on widely different bacterial species to address issues related to the evolutionary pathways that bacteria have followed. Genomic scale sequencing is a highly competitive field and inevitably resulted in different institutes sequencing closely related bacteria. At the time of writing two *Salmonella* genomes, *S.* Typhimurium LT2 (McClelland *et al.*, 2001) and *S.* Typhi CT18 (Parkhill *et al.*, 2001) have been completed with 15 other sequencing projects still in progress (http://www.sanger .ac.uk/Projects/Salmonella) ;(http://genome.wustl.edu/projectts/bacterial/). Comparative genomics of the housekeeping and 16s RNA regions of these species have revealed that they are too closely related at the DNA level to be considered as separate species, with between 96% and 99% similarity for the strains investigated so far (Edwards, Olsen, and Maloy, 2002). As a comparison *Salmonella* species share only 80% median DNA homology with the closely related *E. coli* (McClelland and Wilson, 1998;McClelland *et al.*, 2001). This has resulted in the re-classification of *Salmonella* species with currently only 2 different species recognised, *Salmonella bongori*, which consists of former subspecies V, and *Salmonella enterica* which consists of the remaining seven subspecies (Selander *et al.*, 1990;Boyd *et al.*, 1996;Chan *et al.*, 2003). Although highly homologous at the DNA level the different serovars differ greatly in their ability to infect a variety of hosts and in the severity of disease that ensues, as described below.

1.1.2 Host Specificities and Disease Spectrums

Some *Salmonella* serovars are generalists, infecting a wide range of hosts, others are host adapted, infecting only a few species with the remainder being host specific, infecting only a single host. It is this difference that has spurred an enormous amount of work into the pathogenesis of the different serovars to try and locate the virulence factors which determine host specificity or lack of, and therefore identify targets for vaccine or antibiotic strategies (Morgan *et al.*, 2004).

Salmonella enterica serovar Typhi, henceforward referred to as *S.* Typhi is the principle causative agent of typhoid fever and is host specific to humans and chimpanzees (Edsall *et al.*, 1960). Annually there are approximately 16 million cases of typhoid fever worldwide (although the majority of cases are in the underdeveloped world), which results in around 500,000 fatalities (Pang *et al.*, 1995) (Pang *et al.*, 1998). Symptoms include nausea, vomiting and prolonged fever. The main source of *S.* Typhi infection is through infected water, or through food washed or irrigated with contaminated water. *S.* Typhi infection can be spread by chronic carriers, especially through those who work in food related industries, usually through individuals who have received a low inoculum and harbour an asymptomatic infection (Goldberg and Rubin, 1988). Post ingestion of an infectious dose of *S.* Typhi, which in human volunteers ranges from a thousand to a million organisms (Hornick, 1970;Hornick *et al.*, 1970a;Hornick *et al.*, 1970b), it survives the low pH of the gastric contents and continues on to the lumen of the small intestine. Salmonellae primarily invade the intestinal mucosa through the M cell lining of the Peyer's patches (Carter and Collins, 1974; Hohmann, Schmidt, and Rowley, 1978; Kohbata, Yokoyama, and Yabuuchi, 1986; Jepson and Clark, 2001) where they are able to migrate to the mesenteric lymph nodes and replicate. From the lymph nodes *S.* Typhi enters the bloodstream where it is widely disseminated to systemic sites such

as the spleen, liver, bone marrow, gall bladder and Peyer's patches of the terminal ileum, leading to inflammation, ulceration and necrosis (Everest *et al.*, 2001). The host cellular responses and the ability of *S.* Typhi to evade these responses will be discussed later.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a generalist and able to cause disease in a number of animals and humans. In humans, *S.* Typhimurium was until recently the most common cause of food poisoning in the USA and UK. Symptoms in humans include abdominal pain, nausea and diarrhoea, which can last for longer than a week depending on the challenge dose (Hornick *et al.*, 1970a). However, as with most infections, it can be more serious in the immunocompromised patient, the young and the elderly, and in such cases it does require antibiotic treatment. AIDS patients are approximately 20 times more likely to contract symptomatic salmonellosis than the general population (Sperber and Schleupner, 1987). As the nomenclature suggests *S.* Typhimurium is also capable of infecting a murine host, where it causes a systemic disease similar to that seen with a *S.* Typhi infection in humans reviewed by (Santos *et al.*, 2001). It is for this reason that *S.* Typhimurium infection of mice has provided us with a model of *S.* Typhi infection, which itself does not infect small animals. *S.* Typhimurium infection of susceptible mouse lineages such as the BALB/c mouse are used, and these animals show signs of disease such as ruffled fur and reduced body temperature between four and eight days post oral infection, however diarrhoea does not occur.

S. Enteritidis is the most common cause of *Salmonella* related food poisoning cases reported in the U.K. and elsewhere in the world. The major reason for this is due to its ability to cause oviduct infections of chicken flocks, which are themselves usually asymptomatic, but leads to contamination of egg surfaces. Eggs can be contaminated

through cracks in the shell or transovarally from an infected oviduct to the yolk prior to shell formation (Snoeyenbos, Smyser, and Van Roekel, 1969). Worldwide the incidence of acute gastroenteritis as a result of *Salmonella* infection is estimated at 1.3 billion cases per year, resulting in about 3 million deaths (Pang *et al.*, 1995) and huge economic implications.

Salmonella enterica serovar Pullorum and *Salmonella* enterica serovar Gallinarum are both predominantly fowl specific, although *S.* Pullorum infections in primates have been reported (Ocholi, Enurah, and Odeyemi, 1987). They do however cause distinct fowl diseases, with *S.* Pullorum causing dysentery (pullorum disease) and *S.* Gallinarum causing fowl typhoid (Shivaprasad, 2000). Pullorum disease specifically affects young chicks and poults, forming characteristic lesions in the lungs and necrosis of the liver and spleen, and has a high mortality rate. As a fowl specific pathogen it is not clinically important to humans but continues to cause huge economic loss worldwide. Live chicken vaccines are currently available which confer protection against both *S.* Enteritidis and *S.* Gallinarum. Based on a rough strain of Gallinarum, and therefore safe for use in the food chain, the Nobilis SG 9R vaccine is genetically stable with little chance of reversion (www.safe-poultry.com/NobilisSG-9R). The introduction of this vaccination program within the UK has markedly reduced the number of foodborne cases of *S.* Enteritidis in humans.

S. Bongori are associated with disease or carriage in cold blooded organisms, but incidences of human infections have been reported (Pignato *et al.*, 1998;Nastasi, Mammina, and Salsa, 1999).

With sequencing of at least one representative of all of the species listed in this section either already completed or currently in progress, there will soon be a wealth of comparative genomic data available to the *Salmonella* community. It is hoped that this

data will help to elucidate the genetic differences between species which drives them to be host specific or otherwise. Such genes may make good vaccine or antimicrobial targets, but also from an evolutionary aspect we should be able to dissect how and when these differences occurred.

1.2 Pathogenicity of Systemic *Salmonella* Infection

This section will attempt to address the current perspectives on *Salmonella* Typhi infection in humans. Much of the theory discussed in this section has been extrapolated from observations of *S.* Typhimurium infection in the murine typhoid model. It will discuss the virulence properties of the bacterium itself as well as the interaction of the bacteria with the host cells.

1.2.1 The Intestinal Epithelial Surface

For successful progression of a systemic infection and colonisation of the host after oral infection; penetration of the intestinal epithelium is an important step (Madara *et al.*, 1990). The epithelial surface of the small intestine is a mixed cell population consisting of goblet cells and Paneth cells, with the greatest proportion being columnar absorptive epithelial cells (enterocytes) (Finlay and Brumell, 2000;Sansonetti, 2004). Enterocytes form a polarised barrier, the apical surface of which contains microvilli. A dense coat of glycoproteins, the glycocalyx, reduces access of bacteria to the epithelial surface (Neutra, 1999) (Maury *et al.*, 1995). Goblet cells secrete mucous which forms the mucous layer coating of the epithelial surface acting as an entrapment device. Paneth cells secrete an array of antimicrobial compounds into the intestinal lumen (Ouellette, 1997) including cryptdins, a family of short hydrophobic peptides. In addition to this, intestinal peristalsis and the competition for adherence sites from the commensal microbiota forms a comprehensive mechanism of defence against pathogenic bacteria

(Sansonetti, 2002). As well as barrier protection, the intestinal mucosa has immunological protection provided by gut associated lymphoid tissues (GALT) such as isolated lymphoid follicles, the appendix, mesenteric lymph nodes (MLN) and the Peyer's patches (PP) (Hein, 1999). Peyer's patches are most abundant in the terminal portion of the ileum, near the entrance to the large intestine, and are of particular interest. They are dome shaped follicles covered by follicle associated epithelia (FAE), which also consist mainly of columnar epithelia (90%) (Owen and Ermak, 1990), but contain few goblet cells resulting in a limited layer of mucous, but do contain microfold (M) cells (5%) (Owen and Jones, 1974) unique to the FAE. M cells are derived from regular villous epithelial cells, but themselves lack a regular microvilli and produce very little glycocalyx (Owen, 1994). They do however possess high endocytic activity which allows them to sample the intestinal environment and in particular allows translocation of particulate antigen matter to underlying lymphoid tissue (Ochman *et al.*, 1996;Gebert and Jepson, 1996;Kraehenbuhl and Neutra, 2000). This antigen sampling allows adaptive immunity at the mucosal surface but also labels them as preferred targets for invading pathogens.

1.2.2 Adhesion to and Invasion of Intestinal Epithelia.

S. Typhimurium can adhere to and invade both M cells and columnar epithelial cells of mice, although they appear to have a preference for M cells. About 25% of *Salmonella* can be found associated with Peyer's patches (Carter and Collins, 1974), but Peyer's patches make up such a small proportion of the intestinal epithelia, that this amounts to a preference, with invasion of the M cells occurring at early time points (Hohmann, Schmidt, and Rowley, 1978).

Initial attachment of *S.* Typhimurium to the enterocyte or M cell is mediated through fimbriae or pili, including type 1 fimbriae (fim), plasmid encoded fimbriae (PE), long polar fimbriae (*lpf*) and thin aggregative fimbriae (curli) (Baumler, Tsolis, and Heffron, 1996a). The fimbriae type used appears to be dependent on the cell type or animal species, which indicates binding to a specific host receptor. For instance Fim mediate adhesion to HeLa cells *in vitro* but not T84 or Hep-2 cell lines (Baumler, Tsolis, and Heffron, 1997). Binding to M cells is thought to be mediated by the *lpf* fimbrial operon, although the host receptor for this adhesion remains unknown (Baumler, Tsolis, and Heffron, 1996b).

Due to the difficulties in culturing M cells *in vitro* the majority of *Salmonella* invasion studies have been carried out using the relatively easily manipulatable polarised enterocytes such as Caco-2 and T84, a non intestinal cell line. There are essentially two major mechanisms of bacterial internalisation into non-phagocytic cell types like these (Sansonetti, 2002). The zippering process seen in bacteria such as *Yersinia* species where high affinity binding of a bacterial protein to a host adherence molecule results in bacterial envelopment by the host cell membrane, or the trigger process where the invading bacteria trigger massive cytoskeleton changes in the host cell at the site of contact, resulting in a ruffling process and internalisation of the bacteria in a macropinocytic vacuole (reviewed by (Sansonetti, 2001)). After the initial adhesion process, *Salmonella* species utilise a trigger process mediated by a type three secretion system (TTSS) (Collazo and Galan, 1997). Intracellular *Salmonella* are detected 30 minutes post infection with free bacteria detected on the basolateral side of a monolayer after two hours (Jones and Falkow, 1996). Unlike enterocytes, M cells do not appear to return to normal following *S.* Typhimurium invasion (Jepson and Clark, 2001). Bacterial invasion can lead to M cell death, causing adjacent enterocytes to be shed and

allowing luminal bacteria to pass directly into the lamina propia (Jones, Ghori, and Falkow, 1994).

TTSS are complex molecular machines found in a large number of Gram negative pathogens of humans, animals and plants to translocate virulence proteins into the host cell cytoplasm (Hueck, 1998). They are usually located within pathogenicity islands or on plasmids, and encode for proteins with a number of functions including structural, regulatory, chaperones and secreted proteins (Lostroh and Lee, 2001). The structure, function and regulation of TTSS has been subject to much discussion which is still ongoing. Generally the TTSS apparatus are highly conserved and share similarities with the outer membrane component of type II secretion systems and with the flagellar basal body (Kubori *et al.*, 1998). This apparatus consists of a cytoplasmic bulb followed by a disk like structure that spans the cytoplasmic and periplasmic membranes. A needle structure crosses the previous domains and extends outside the outer membrane. Contact of this structure with the host cell initiates translocation of the secreted proteins. The translocated proteins, effector proteins, are secreted through the scaffolding structure and 'injected' into the host cell. The effector proteins either interfere with or modify host cell signal transduction, cytoskeletal architecture, membrane trafficking and cytokine gene expression. Unlike the apparatus proteins, the effector proteins differ from one TTSS to another and indeed between salmonellae (Wallis and Galyov, 2000;Bakshi *et al.*, 2000).

Salmonella pathogenicity island-1 (SPI-1) is a large genetic element, thought to be acquired before speciation of *Salmonella* (Ochman and Groisman, 1996), present in all serovars of *S. enterica* as well as in *S. bongori*. SPI-1 is the best characterised of the twelve *Salmonella* pathogenicity islands. It is approximately 40kb in size and is located at 63 minutes on the *S.* Typhimurium chromosome (Mills, Bajaj, and Lee, 1995), with

an overall GC percentage of 42% as opposed to the average 53% of the *S.* Typhimurium backbone, a good indication that its ancestry lies elsewhere. SPI-1 contains 39 genes (Lostroh and Lee, 2001), encoding various components of a TTSS (TTSS-1), with additional effector proteins of this secretion system encoded elsewhere on the chromosome.

SPI-1 mutants are not attenuated after infection of mice via the intraperitoneal route (IP) (Ochman *et al.*, 1996;Galan, 1996a;Galan, 1996b) but are unable to invade enterocytes *in vitro* and have a twenty fold greater LD50 than a wild type *S.* Typhimurium strain in mice inoculated orally (Lucas and Lee, 2001). This implies a role for SPI-1 in colonisation of the gut, an important stage for initiation of systemic infection, but does not play a role in systemic infection per se.

The expression of genes encoding the TTSS-1 are not constitutive (Hueck, 1998). *In vitro* conditions known to induce TTSS-1 expression include high osmolarity, low aeration, and basic pH (Ernst, Dombroski, and Merrick, 1990;Lee and Falkow, 1990), which reflects the physiological conditions experienced by a pathogen, *in vivo,* after oral infection. The expression of genes encoding TTSS-1 apparatus and the majority of its effectors requires HilA (Bajaj, Hwang, and Lee, 1995), a transcription factor, encoded on SPI-1. Invasion of *S.* Typhimurium into epithelial cells is totally dependent on functional HilA (Lee, Jones, and Falkow, 1992;Bajaj, Hwang, and Lee, 1995;Bajaj *et al.*, 1996) . SPI-1 encodes four additional transcriptional regulators, SprB, HilC, HilD (Lucas and Lee, 2001) and InvF (Darwin and Miller, 1999;Eichelberg and Galan, 1999), which co-regulate the TTSS-1 in a cascade of transcriptional activation, in which HilD, HilA and InvF act in sequence to activate the TTSS-1 *in vivo.* Expression of HilA itself is regulated by SirA (*Salmonella* Invasion Regulator) (Johnston *et al.*, 1996) and a mutation in *sirA* reduces invasiveness of *S.* Typhimurium. SirA is a highly conserved

protein amongst Gram negative bacteria (Johnston *et al.*, 1996), which unlike the other transcriptional regulators involved in regulation of TTSS-1, is not located within SPI-1, but rather at 42 minutes on the chromosome.

After transcriptional activation of TTSS-1, the TTSS apparatus forms. These proteins are encoded for by two sets of genes, *inv-spa* and *prg-org* (Ginocchio, Pace, and Galan, 1992;Groisman and Ochman, 1993;Jones, Ghori, and Falkow, 1994;Eichelberg, Ginocchio, and Galan, 1994;Kaniga, Bossio, and Galan, 1994). *inv-spa* and *prg-org* are located within SPI-1 but are separated by effector proteins and HilA. The *prg* (PhoP-repressed) genes are themselves regulated by the two component regulator *phoPQ* (Miller, 1991;Behlau and Miller, 1993;Kimbrough and Miller, 2000) adding an additional level of complexity to the regulatory pathway of SPI-1. *phoPQ* expression is activated by low concentrations of divalent cations. *prg* gene expression is derepressed under high concentrations of Mg^{2+}, such as those present in the extracellular environment enabling production of the *prg* proteins which form the syringe like structure through which effector proteins are translocated (Kimbrough and Miller, 2000).

Identification of secreted effector proteins for TTSS has been an intensive area of research in recent years, and the TTSS of *Salmonella* are no exception. Currently there are 13 known effector proteins for TTSS-1 (Waterman and Holden, 2003). Examples of SPI-1 encoded effector proteins are SptP (*Salmonella* protein tyrosine phosphatase) (Kaniga *et al.*, 1996;Fu and Galan, 1998a;Fu and Galan, 1998b), SipA (*Salmonella* invasion protein) (Zhou, Mooseker, and Galan, 1999), SipB, SipC (Raffatellu *et al.*, 2005) and AvrA (avirulence factor A) (Hardt and Galan, 1997). SptP possesses C terminal phosphotyrosine phosphatase activity and an N terminal that shares homology with YopE of *Yersinia* species. Both domains are involved in disruption of the actin

cytoskeleton of the epithelial cell, but SptP is not required for invasion and only demonstrates a subtle defect in comparison to wild type *S.* Typhimurium in mice (Fu and Galan, 1999). The Sip proteins show high levels of homology to the Ipa effectors of *Shigella* species which are required for invasion (Menard, Sansonetti, and Parsot, 1993;Kaniga, Trollinger, and Galan, 1995;Kaniga *et al.*, 1995;Hermant *et al.*, 1995). SipA binds directly to actin in the host cell, inhibiting depolymerisation of the actin filaments and decreasing the critical concentration of actin (Hayward and Koronakis, 1999;Zhou, Mooseker, and Galan, 1999;Bourdet-Sicard and Tran, 1999;Galan and Fu, 2000). This suggests a role in host cytoskeletal rearrangement for SipA. SipB and SipC appear to have dual functions as translocators for other effector proteins (SptP, AvrA) as well as being delivered to the host cells to act as effectors themselves (Collazo and Galan, 1997), where SipB binds to caspase-1 initiating apoptosis (Hersh *et al.*, 1999) whilst SipC plays a role in actin bundling (Hayward and Koronakis, 1999;McGhie, Hayward, and Koronakis, 2001). Examples of TTSS-1 effector proteins not encoded within SPI-1 are SopE and SopE2. *sopE* is located within a P2-like prophage, and is not present in all *Salmonella* serovars, unlike *sopE2* which appears to be broadly distributed in salmonellae (Bakshi *et al.*, 2000). SopE and SopE2 are 69% identical and are both highly efficient guanine nucleotide exchange factors (Hardt, Urlaub, and Galan, 1998;Rudolph *et al.*, 1999) that can activate host cell Rho family GTPases (Stender *et al.*, 2000;Bakshi *et al.*, 2000;Friebel *et al.*, 2001). SopE can activate Cdc42 and Rac1 but SopE2 activates only Cdc42 (Friebel *et al.*, 2001). Cdc42 and Rac1 control actin polymerisation and production of pro-inflammatory cytokines like IL-8 via ERK, JNK and p38 (Friebel *et al.*, 2001). Induction of these pro-inflammatory cytokines contributes to the diarrhea associated with *Salmonella* induced enteritis (Zhang *et al.*, 2002). Actin-cytoskeleton reorganisation induced by *Salmonella* is reversible, unless

functional SptP is absent (Fu and Galan, 1998b;Galan and Fu, 2000;Murli, Watson, and Galan, 2001;Lin, Le, and Cowen, 2003) where cytoskeletal disruption continues hours post infection. SptP acts as a GTPase-activating protein (GAP) for Rac-1 and Cdc-42 (Galan and Fu, 2000).

The overlap in function and limited effect that deletions in individual effector proteins have on invasion indicates a degree of redundancy for some of the effector proteins of TTSS-1, but ensures that the goal of efficient internalisation is achieved, and in the early stages of systemic infection the intestinal epithelial barrier has been breached.

1.2.3 Intracellular Survival of *S.* Typhimurium

As the preferential epithelial cell type for invasive *Salmonella* species, we will consider the sequence of events post invasion of M cells. M cell invasion by *Salmonella* results in destruction of the M cell and sloughing of the FAE. The epithelial damage induced by invasion of M cells may correlate with intestinal ulcerations seen in *S.* Typhi infection of humans (Jones and Falkow, 1996). SPI-1 mutants cannot cause the degree of cytotoxicity and FAE destruction seen with wild type bacteria (Galan and Curtiss, III, 1989;Penheiter *et al.*, 1997;Clark, Hirst, and Jepson, 1998;Jepson and Clark, 1998), but there is evidence that *S.* Typhimurium strains deficient in SPI-1 can still invade the mouse intestine, spread to the liver and spleen and cause lethal disease. Vazquez-Torres *et al.*, 1999 suggested that non invasive strains of *S.* Typhimurium may cross the intestinal barrier and disseminate from the intestine by invading $CD18^+$ phagocytes such as macrophages and dendritic cells. They suggest that this is through the ability of mucosal macrophages or dendritic cells (DC) to transmigrate bidirectionally across the epithelial barrier, or may even extend processes across enterocyte tight junctions to sample the intestinal lumen. This study did not specifically identify the type of $CD18^+$ phagocyte responsible for transportation of the bacteria but (Rescigno *et al.*, 2001)

suggest that DC cells could be involved in M cell- independent transport of non invasive *Salmonella* from the intestine. In agreement with (Vazquez-Torres *et al.*, 1999) they suggest the possibility that DCs breach the intestinal epithelia, phagocyte luminal bacteria and bring them across the gut epithelium. This has raised the question as to whether DCs are the only type of intestinal phagocyte that can sample intestinal bacteria (Lencer and Neutra, 2000).

Knowing that SipB stimulates caspase-1 killing of macrophages (Hersh *et al.*, 1999), (Monack *et al.*, 2000) investigated the role of caspase-1 in *S.* Typhimurium oral infection of mice using a caspase-1 knock out mice strain, which showed less colonisation, inflammation and apoptosis of Peyer's patches. However, M cell invasion and cytotoxicity was still present and suggests an alternative method to cytotoxicity other than the caspase-1 pathway. The cytotoxic effects of *Salmonella* to the M cells expose the invading bacteria to the resident macrophages and dendritic cells, which phagocytose the bacteria. *Salmonella* have evolved various strategies to survive within host cells and combat the harsh intracellular environment. As previously mentioned upon phagocytosis by macrophages, SipB can initiate apoptosis by the caspase-1 signalling pathway. However, to evade the host immune responses and enable local and systemic dissemination, virulent *Salmonella* are able to survive and replicate within the phagocyte. How the balance between apoptotic killing of and the survival within macrophages is achieved is unknown, but it is thought to involve a SPI-1 to SPI-2 switch.

Immediately upon internalisation within the macrophage (or other host cell), *S.* Typhimurium is localised within a membrane compartment known as the *Salmonella* containing vacuole (SCV) (Steele-Mortimer *et al.*, 1999), although variation in the SCV

and it's trafficking differs between cell types. The initial vacuole has early endocytic markers on the membrane, which between 10 and 20 minutes post invasion are replaced by lysosome associated membrane proteins (LAMPs)(Steele-Mortimer *et al.*, 1999). Intracellular bacterial replication experiences an initial lag but recovers and is accompanied by the formation of Sifs (*Salmonella* induced filaments) (Garcia-del Portillo, Foster, and Finlay, 1993;Garcia-del Portillo *et al.*, 1993;Garcia-del Portillo and Finlay, 1994), in epithelial, fibroblast and macrophage cell types (Martinez-Moya *et al.*, 1998;Knodler *et al.*, 2002), although Sifs have not been identified *in vivo*. The endocytic pathway of eukaryotic cells has evolved to recycle or degrade molecule from the cell surface or external milieu. The SCV prevents degradation by blocking assembly of a functional hydrolytic phagolysosome, although there are multiple interactions between the SCV and the endocytic pathway (Knodler *et al.*, 2003). A number of these interactions as well as regulation of the SCV itself have been assigned to a number of different effector proteins of the second TTSS of *Salmonella* SPI-2 (Waterman and Holden, 2003).

Salmonella pathogenicity island 2 (SPI-2) is almost 40kb in length and encodes for more than 40 genes including a two component regulator (SsrAB) (Garmendia *et al.*, 2003) and a second TTSS (TTSS-2) (Shea *et al.*, 1996;Hensel *et al.*, 1997). TTSS-2 consists of a 26kb region located at the 30 centisome region and is organised into four operons, regulatory, structural I, structural II and effector/chaperone (Shea *et al.*, 1996;Hensel *et al.*, 1998;Cirillo *et al.*, 1998). Currently there are 23 SPI-2 effector proteins either encoded for within SPI-2 itself or elsewhere on the *Salmonella* chromosome (Waterman and Holden, 2003). SpiC was the first of the SPI-2 effector proteins to be characterised, with a mutation in SpiC resulting in poor intramacrophage survival due to an increase in the SCV fusing with endosomes or lysosomes (Uchiya *et*

al., 1999). Recently a novel host cell protein, TassC, has been identified as the target for SpiC (Lee, Zareei, and Daefler, 2002). SifA is a non-SPI-2 encoded protein regulated by SsrAB, mutants of which have a replication defect in macrophages due to loss of the vacuolar membrane, exposing the bacteria to cytosolic killing (Miao and Miller, 2000;Miao, Freeman, and Miller, 2002). SifA seems to be important for the recruitment of LAMP containing vesicles to the bacterial microcolony and fusion of these with the SCV (Ruiz-Albert *et al.*, 2002). The cellular target for SifA remains unknown. There are currently a number of groups investigating the role of SPI-2 genes and formation and survival within the SCV, which is beginning to dissect the signalling pathways involved but is beyond the scope of this thesis.

Other non SPI-2 mechanisms are also essential for intramacrophage survival, including RecABC involved in repair of damage caused by superoxide (Buchmeier *et al.*, 1993), the complex PhoPQ regulatory pathway (Garcia, Soncini, and Groisman, 1994), the acid tolerance response involving *atp* and *fur* (Foster, 1991;Garcia-del Portillo, Foster, and Finlay, 1993;Foster, 1993;Gupta *et al.*, 1995;Hall and Foster, 1996;Riesenberg-Wilmes *et al.*, 1996;Foster and Moreno, 1999), and the alternative sigma factor, σ^E (Humphreys *et al.*, 1999). σ^E forms the basis of this thesis and will be discussed in great detail later.

1.2.4 Other Virulent Related Structures of *Salmonella* Species

Many non-typhoid species of *Salmonella* carry large virulent associated plasmids (50-90kb) important for systemic infection (Gulig, 1990;Guiney *et al.*, 1995). The virulence plasmid of *S.* Typhimurium, pSLT, is carried by 90% strains, and is self transmissible (Gulig *et al.*, 1993). A highly conserved 8 kb operon of five genes designated *spvABCD* and *spvR* is found on all virulence plasmids from all serovars examined so far (Gulig *et al.*, 1993), with *spvR* regulating the expression of the *spv* genes (Guiney *et al.*, 1995).

SpvR itself is regulated by RpoS, an alternative sigma factor associated with stationary phase survival (Heiskanen, Taira, and Rhen, 1994). SpvA is the negative regulator of the operon (Abe *et al.*, 1994). A role for the *spv* genes in virulence is still being elucidated. If the virulence plasmid is cured then there is a loss of virulence which is restored by the introduction of the *spv* genes (Gulig *et al.*, 1993). However, no relationship between *spv* gene expression and virulence has yet been resolved.

As previously discussed five pathogenicity islands for *Salmonella* have been identified so far. SPI-3 is a 17kb insertion at a site similar to islands in EPEC and uropathogenic *E. coli*, containing ten ORFs (Marcus *et al.*, 2000). Two of these ORFs form an operon, mgtCB, regulated by PhoPQ, which play a role in virulence and macrophage survival (Blanc-Potard and Groisman, 1997) (Blanc-Potard *et al.*, 1999). SPI-4 is a 25kb insertion, with 18 putative ORFs which has been proposed to form a type I secretion system (Wong *et al.*, 1998). The main function of SPI-4 remains unknown. SPI-5 encodes genes which have a minimal impact on systemic infection, but has an attenuated effect on secretion in an enteritis model, including *pipD*, *sopB* and *pipB* (Wood *et al.*, 1998).

1.3 The Immune Response to Systemic *Salmonella* Infection

The immune response to bacterial infection involves a concerted effort from the innate and adaptive systems. The type of immune response seen in the presence of systemic *Salmonella* infection appears to be dependent on the progression and localisation of infection. Here we discuss the host immune responses to systemic *Salmonella* infection.

1.3.1 Innate Immunity

By definition innate immune responses are those that are not acquired after exposure to an infectious agent. Methods of innate immunity to *Salmonella* infection prior to

systemic invasion include, peristalsis of the gut, complement, opsonins, antimicrobial peptides, cilia, mucin, lysozyme, and the intestinal cell glycocalyx. The innate response includes phagocytic and non-phagocytic cells including neutrophils, macrophages and natural killer cells, which aim to eradicate infection through phagocytosis and killing of bacteria, a difficult prospect when faced with a systemic *Salmonella* infection that is capable of surviving in the intracellular environment of host cells, including the professional phagocytes. A decisive factor for the potential of macrophages to kill *S.* Typhimurium is the expression of a functional *Nramp*1 (Natural resistance-associated macrophage protein) molecule (Govoni and Gros, 1998;Canonne-Hergaux *et al.*, 1999). *Nramp*1 is a transmembrane protein that is expressed in macrophages, encoded for by *bcg* and is structurally related to cation channels (Pitel *et al.*, 1994). The presence of a mutant form of this gene in some mouse strains (ity^S) (Robson and Vas, 1972;Plant and Glynn, 1974) reduces the ability of their macrophages to kill *S.* Typhimurium, thus they are unable to control the infection and die as a consequence of exponential growth of *S.* Typhimurium in the liver and spleen.

For *Salmonella* the major bacterial factor eliciting the innate immune response is LPS (Wyckoff, Raetz, and Jackman, 1998) (Freudenberg *et al.*, 2001). Binding of LPS to macrophage membranes sensitises them to activation by IFN-γ. Opsonisation of bacteria with the serum glycoprotein LPS-binding (LPS-B) protein mediates the adhesion of coated bacteria to macrophages. A third innate response to LPS is to the soluble form of LPS found within the serum. LPS-B binds this and forms a complex recognised by the CD14 receptor (Wright *et al.*, 1990;Schutt, 1999) resulting in the secretion of TNF-α. CD14 is a glycosylphosphatidylinositol linked membrane protein, and binds structurally unrelated proteins. Small amounts of LPS are beneficial to the host as they induce a host inflammatory response, mediated by TNF-α, necessary for recruitment of

neutrophils to endothelial cells and eventually their migration to sites of infection (Raetz, 1993). Interestingly a mutant allele of Toll like receptor 4 (TLR4) has been identified in mice unresponsive to LPS (lps^d), and subsequently TLR4 has been suggested as one of the host recognition molecules for bacterial LPS (Poltorak *et al.*, 1998a) (Poltorak *et al.*, 1998b). TLR4 polymorphism can occur within a species which may account for differences in LPS sensitivities observed (Smirnova *et al.*, 2000).

Another PAMP (pathogen associated molecular patterns), molecules that are conserved on microbial pathogens and not represented in the host (Janeway, Jr. and Medzhitov, 2002), is the bacterial flagellin. In models of mucosal infection the production of functional flagella is a virulence factor, as is the case with *S.* Typhimurium, where mutation of the flagella genes attenuates the strain (Schmitt *et al.*, 2001). Mammalian TLR5 is now known to recognise bacterial flagellin, and that activation of the receptor stimulates TNFα production via NF-$_\kappa$B (Hayashi *et al.*, 2001;Smith *et al.*, 2003). This explains the observed innate response to *S.* Typhi flagellin, which results in induction of proinflammatory cytokines and monocytes (Wyant, Tanner, and Sztein, 1999).

1.3.2 Acquired Immunity

Although the innate mechanisms of the immune response are highly effective in restricting initial growth of *S.* Typhimurium, these mechanisms alone fail to eliminate the bacterium from the host. Only the generation of a specific lymphocyte response allows the eventual eradication of bacteria, and provides increased protection or 'memory' against further infection.

Protection against future *Salmonella* infection and the ability to clear late infection is the result of both the cellular and humoral arms of the acquired immune response (Mastroeni and Menager, 2003). Following immunisation with protective live attenuated *Salmonella* vaccines long lasting immunological memory develops in

animals and humans (Mastroeni and Menager, 2003). The elicited serum and mucosal responses are directed towards a broad spectrum of antigens including LPS, Vi, porins, outer-membrane proteins, lipoproteins, heat shock proteins, flagella and fimbriae (Kuusi *et al.*, 1979;Kantele, Arvilommi, and Jokinen, 1986;Harrison *et al.*, 1997). These responses are predominantly of the T_H1 type determined by the production of IL-2 and IFN-γ (Harrison *et al.*, 1997). Class Ib $CD8^+$ T cells capable of lysing *Salmonella* infected target cells also appear after vaccination (Lo *et al.*, 1999). Long term protection against *Salmonella* requires the antigen specific recall of immunity, with the involvement of antibodies, T cells and cytokines (Mastroeni and Menager, 2003). In experimental models, antibodies or immune T cells alone can protect against secondary challenge only when the bacteria is modestly virulent or the host is innately resistant (Eisenstein, Killar, and Sultzer, 1984;Xu *et al.*, 1993a;Xu *et al.*, 1993b). This has been suggested as a reason for the moderate efficacy of human typhoid vaccines based on the Vi polysaccharide antigen of *S.* Typhi (Acharya *et al.*, 1987;Klugman *et al.*, 1996). Such vaccines can induce antibody responses but are believed to be incapable of inducing T_H1 immunity (Thatte, Rath, and Bal, 1993;Harrison *et al.*, 1997).

1.4 Bacterial Stress Responses

Bacteria have evolved a number of stress responses to sense changes in their microenvironment which may be detrimental to their survival. For pathogenic bacteria capable of surviving within a variety of warm and cold blooded hosts, as well as out with of the host, combinations of these stresses can be encountered at any given time, depending on the stage in the bacterial life cycle. The stress response induces a number of transcriptional changes, most of which utilise alternative sigma factors and two component regulators. The resulting changes in protein levels either combat the stress directly or repair and replace any damaged intracellular components as a result of the

stress, thus maintaining homeostasis. Such responses are highly conserved across organisms, and are often compartmentalised into separate pathways so that the response is not global. This is indeed the case for Gram negative bacteria where stress responses are compartmentalised to the periplasm and cytoplasm, although there is a degree of overlap between the two. Here we will discuss the stress responses of Gram negative bacteria, but in particular it will focus on the extracytoplasmic stress response (ESR) and the signalling pathways involved.

1.4.1 Sigma Factors and RNA Polymerase

Before trying to review the nature and complexity of the stress response, we should first examine some of the fundamental knowledge that we already possess regarding sigma factors and their interactions with RNA polymerase. DNA dependent RNA polymerase is a heteromultimeric complex of five essential protein subunits (Archambault and Friesen, 1993;Gross, Chan, and Lonetto, 1996;Zhang *et al.*, 1998;Minakhin *et al.*, 2001) and is the key enzyme of transcription and expression in all living organisms. Four of the subunits $\alpha_2\beta\beta'$ can be co-purified as an associated complex largely known as the core RNA polymerase. Association of the core RNA polymerase with a sigma (σ) subunit forms the holoenzyme, reviewed by (Borukhov and Nudler, 2003). It is the σ subunit that directs RNA polymerase to initiate transcription at specific promoter sites on the DNA (Helmann and Chamberlin, 1988) whilst either the σ factor or any of the other four subunits can interact with transcriptional activator proteins to facilitate promoter recognition. A set of genes can be co-ordinately expressed if their promoter sequences are recognised by a single σ factor. In *E. coli* and *Salmonella* species the primary σ factor, σ^{70}, is known as the house keeping sigma factor. σ^{70} is encoded for by *rpoD* (Nakamura, Osawa, and Yura, 1977), and is responsible for transcription of the

majority of genes expressed during exponential growth, including genes of the essential biosynthetic pathways such as biosynthesis of amino acids, nucleotides, cell wall and membrane components (Lonetto, Gribskov, and Gross, 1992;Paget and Helmann, 2003). In addition to the housekeeping sigma factor, other σ factors known as the alternative sigma factors, direct the transcription of sets of genes, the products of which are required for specific functions (Lonetto, Gribskov, and Gross, 1992) (Ishihama, 2000) (Hengge-Aronis, 2002a). These alternative sigma factors recognise different –10 and –35 promoter sequences. This confers differential promoter specificity to RNA polymerase hence providing transcription regulation. The majority of the sigma factors are highly conserved at the amino acid level, which would be expected due to the shared functions that they possess e.g. binding to the core RNA polymerase, reviewed by (Hughes and Mathee, 1998)

1.4.2 Two Component Regulators

Another common genetic structure involved in sensing an external stress and relaying this information to ultimately result in an altered transcription profile is the two component regulator. Two component regulators exist that respond to a variety of environmental signals including nitrogen, oxygen, osmolarity etc. The basic two component system comprises of a sensor histidine kinase (HK), also called a transmitter protein, and a cognate response regulator (RR) which acts as a transcription factor. The HK and RR are often transcriptionally linked and situated within the same operon. Sensor kinases consist of two domains, an N terminal sensor domain which recognises activation signals (Galperin, Nikolskaya, and Koonin, 2001), and a C terminal autokinase domain (Ninfa and Magasanik, 1986). The HK contains an invariant histidine residue in the autokinase domain that is autophosphorylated via an ATP dependent reaction (West and Stock, 2001). Most two component systems involve a

single Histidine to Aspartate phosphotransfer event, although some do consist of a three step phosphorelay reaction (Stock, Robinson, and Goudreau, 2000). During this reaction the phosphate group is transferred from the Histidine in the autokinase domain to an Aspartate group within the N terminal domain of the RR. This phosphorylation activates the C terminal DNA binding region of the RR and results in differential expression of genes under its control (Stock, Robinson, and Goudreau, 2000). This describes the basic two component regulator but there is a variation of this theme. More complex systems can use a Histidine containing phosphotransmitter (HPt) to relay the phosphate group between HK and RR (West and Stock, 2001). Different signals can be fed into the same signal if the RR can be modified by several sensor domains. Also certain HKs have been shown to interact and phosphorylate RR other than its cognate partner, in a process referred to a cross talk (Stock *et al.*, 1988;Ninfa *et al.*, 1988;Wanner, 1992). An example of this is the PhoB/PhoR system, where in strains lacking PhoR, PhoB activated genes can be induced by glucose and pyruvate through CreC (Wanner and Latterell, 1980). The relevance of such systems is still under investigation because although several examples have now been demonstrated *in vitro* (Ninfa *et al.*, 1988;Fisher *et al.*, 1995;Yaku and Mizuno, 1997) when the non-cognate proteins are present in high amounts usually when the cognate sensor is missing, only a few examples have been described *in vivo* (Kim, Wilmes-Riesenberg, and Wanner, 1996;Silva *et al.*, 1998;Matsubara and Mizuno, 1999;Matsubara *et al.*, 2000). Analysis of the *E. coli* K12 genome revealed the presence of 29 histidine kinases and 32 response regulators (Oshima *et al.*, 2002;Yamamoto *et al.*, 2004).

1.4.3 The Cytoplasmic Stress Response

We will now discuss some of the specific stress response systems which use the genetic structures described to alter transcriptional profile in response to a given stress.

Probably the best characterised of these is the cytoplasmic stress response mediated by an alternative sigma factor, σ^H or σ^{32}, encoded for by *rpoH* (Grossman, Erickson, and Gross, 1984). Originally named the heat shock response, as the original role proposed for σ^H was to reverse the effects of temperature shift on the bacterial cell; loss of function *E. coli* σ^H mutants are only able to grow at temperatures of 20°C or less (Zhou *et al.*, 1988). The *rpoH* regulon is now known to be triggered by any stress that results in damage to the cytoplasmic membrane and a build up of misfolded or damaged proteins within the cytoplasmic space (Bukau, 1993). In *E. coli* the regulation of at least 30 heat shock proteins (HSPs) are under the control of σ^H, with the majority of these encoding for chaperones or proteases involved in protein quality control.

Under non-stressed conditions the physiological level of σ^H is relatively low in comparison with RpoD (Craig and Gross, 1991), and the intracellular level is controlled at four different levels, transcription and translation of the *rpoH* gene, and activity and stability of the σ^H gene. At the transcriptional level, σ^H is regulated by at least 4 promoters (Erickson *et al.*, 1987;Nagai *et al.*, 1990), P1, P3, P4 and P5, with the usage of each promoter dependent upon temperature. At 30°C 90% of transcription is from the P1 and P4 promoters. At increasing temperatures an increase in transcription from the P3 promoter occurs, until at 50°C the P3 promoter is the sole site for transcription (Wang and Kaguni, 1989). The P3 promoter is regulated by the alternative sigma factor σ^E (Erickson and Gross, 1989;Wang and Kaguni, 1989), with the remaining three promoters controlled by σ^D. Two master regulatory proteins are known to fine tune transcription, the P5 promoter is totally dependent on the cAMP receptor protein (CRP) (Kallipolitis and Valentin-Hansen, 1998), whereas the P3 and P4 promoters are negatively regulated by DnaA (Wang and Kaguni, 1989;Nagai *et al.*, 1990). However, heat induction of σ^H mainly occurs at the post-transcriptional level where under non

elevated conditions the secondary structure of the *rpoH* mRNA transcript prevents translation. Thermal melting of the *rpoH* secondary structure enables ribosome binding and initiation of translation (Tilly *et al.*, 1986;Erickson *et al.*, 1987). Under non-stress conditions σ^H is prevented from interacting with the holoenzyme by interaction with the DnaK, DnaJ chaperone machinery (Tatsuta *et al.*, 1998;Tatsuta *et al.*, 2000). Not only does this binding prevent interaction with the holoenzyme it also targets σ^H for degradation by the FtsH protease. Under cytoplasmic stress conditions the DnaK machinery binds to misfolded polypeptides, leaving free σ^H to bind to the holoenzyme.

1.4.4 Stationary Phase and Starvation Stress Responses

Another well characterised example of a bacterial stress response is controlled by the secondary sigma factor, called σ^S or σ^{38}, encoded for by RpoS. It is so called due to its crucial role in stationary phase survival (Lange and Hengge-Aronis, 1991), although it is now recognised as a gene involved in a plethora of regulatory pathways, and is often referred to as the master regulator of the general stress response (Hengge-Aronis, 1993;Muffler *et al.*, 1997). It is referred to as a secondary sigma factor rather than an alternative sigma factor due to its close homology to RpoD and the similarity in promoter sequences that they recognise. RpoS is found amongst the γ branch of the proteobacteria, although with minor variations in roles compared with those described in *E. coli* (Hengge-Aronis, 2002a;Hengge-Aronis, 2002b). When the RpoS stress response is triggered the bacterial cells often undergo a cessation in growth, which allows protection against the given stress, but also confers for cross protection against future stresses. This is the difference between this response and others described in this section, which are usually triggered by a single stress type and results in the induction of proteins that work to nullify the stress and repair damage caused, whereas the RpoS

response works to prevent cell damage. *E. coli* RpoS mutants are highly sensitive to oxidative stress, temperature shift above 50°C, acid pH, ethanol, and prolonged stationary phase survival (Hengge-Aronis, 2002a), but the physiological consequences of induction of the general stress response may not always be the same depending on the stress signal involved, due to the role of co-regulators. There is a large diversity amongst the regulatory factors involved but include H-NS (Robbe-Saule *et al.*, 1997), Fis (Xu and Johnson, 1995a;Xu and Johnson, 1995b) and CRP (Lange, Barth, and Hengge-Aronis, 1993) as ones that occur most often. Homologues of RpoS have been described in a number of bacteria with functional homology described across species. *Salmonella rpoS* mutants show a degree of attenuation *in vivo* (Fang *et al.*, 1992). A degree of this may be due to loss of resistance to acidic conditions or oxidative stress, but in part has been attributed to the role that RpoS plays in regulation of the *spv* virulence genes (Heiskanen, Taira, and Rhen, 1994;Kowarz *et al.*, 1994). Also curli fimbriae in *S.* Typhimurium require RpoS for expression (Romling *et al.*, 1998a;Romling *et al.*, 1998b).

As mentioned the sequence similarity of *rpoS* and *rpoD* has governed that they recognise and bind to similar promoter regions, but no common -35 region has been elucidated for σ^S (Loewen *et al.*, 1998), unlike for the majority of other sigma factors, indicating that this region is unimportant for the recognition and binding of σ^S, or that it requires co-factors. However a -10 promoter region very similar to that for σ^D has been identified (Loewen and Hengge-Aronis, 1994) (Espinosa-Urgel, Chamizo, and Tormo, 1996) and this with the presence of intrinsic curvature (Espinosa-Urgel and Tormo, 1993) has been used as a consensus for σ^S regulated promoters. Some promoters are therefore regulated by both σ^S and σ^D, if a σ^D -35 region is present, which accounts for the degree of overlap between σ^S and σ^D regulated genes (Tanaka *et al.*, 1993).

Protein levels of RpoS are virtually undetectable throughout exponential phase but rise to levels up to 50% of that measured for RpoD in stationary phase (Jishage and Ishihama, 1995). Transcription of *rpoS* is regulated by 4 promoters with the majority of transcription coming from the promoter designated *rpoS*1 (Lange, Fischer, and Hengge-Aronis, 1995). Post transcriptional regulation of *rpoS* also exists where high osmolarity, low temperature and stationary phase stimulates *rpoS* mRNA translation (McCann, Fraley, and Matin, 1993) (Loewen *et al.*, 1993). Secondary structure is the primary determinant controlling translation of *rpoS* mRNA, with the ribosome binding site and initiation codon usually inaccessible within the secondary structure. The RNA binding protein, Hfq, has been shown to disrupt the secondary structure, resulting in an increase in translation of RpoS (Brown and Elliott, 1996). The reverse of this is mediated through the DNA binding protein H-NS, which has been associated with a repressive effect on RpoS translation during exponential phase (Barth *et al.*, 1995). The stability of translated RpoS is also modulated by the protease ClpXP which is responsible for the instability of RpoS during exponential phase (Schweder *et al.*, 1996). RpoS becomes much more stable during stationary phase and this decrease in degradation is not through a decrease in the protease, but through a protective role assigned to the chaperone DnaK (Tatsuta *et al.*, 2000).

One of the most common stresses to bacteria such as *Salmonella* which are capable of surviving within a number of hosts as well as within the environment is starvation for essential nutrients such as a carbon source (Spector, 1998). A distinction must be made between cells in stationary phase and those that are starved. Stationary phase cultures are those that have stopped growing following exponential growth in non –limiting media, as opposed to starved cultures which have stopped growing due to the exhaustion or absence of at least one nutrient (Spector, 1998). Stationary phase cultures

normally achieve a much higher cell density than starved cells, with starvation stress a much better defined stress. The response of *S.* Typhimurium to starvation stress has been studied extensively. The two major regulatory proteins of the starvation response are RpoS and CRP, both of which exhibit increased expression in starved cultures (Foster and Spector, 1995). CRP is the major regulator and acts as both a repressor and activator of genes in the starvation response regulon (O'Neal *et al.*, 1994).

To overcome carbon starvation a number of physiological changes occur within the cell. One of these is the induction of new or higher affinity transport systems to scavenge the surrounding environment for the missing nutrient (Spector, 1998). Examples of these are the *csiA* and *csiG* loci. Another important physiological change is a switch from anabolic metabolism to catabolic metabolism (Spector *et al.*, 1986).

1.4.5 RpoN

An alternative sigma factor found in many bacterial species is σ^N (σ^{54}), encoded for by *rpoN*, and also referred to as *ntrA* (nitrogen regulatory protein A). This sigma factor differs in the others described here in that it is involved in diverse metabolic functions such as nitrogen fixation, nitrogen assimilation, C4-dicarboxylate transport, hydrogen metabolism, degradation of aromatic compounds and pilin biosynthesis and also recognises a specific -12 and -24 promoter consensus region rather than the usual -10 and -35 (Merrick, 1993). The mechanism by which σ^N initiates transcription is also different as the σ^N-holoenzyme binds to the promoter forming a closed promoter complex (Morett and Buck, 1989;Popham *et al.*, 1989;Sasse-Dwight and Gralla, 1990), and requires an enhancer binding protein for isomerisation to an open promoter complex (Su *et al.*, 1990;Rippe *et al.*, 1997). Enhancer binding proteins generally bind to sites upstream of the promoter and make contact with the σ^N-holoenzyme complex

through DNA looping (Su *et al.*, 1990;Rippe *et al.*, 1997). The enhancer protein must be able to hydrolyse ATP or GTP (Popham *et al.*, 1989;Weiss *et al.*, 1991) to catalyse the isomerisation reaction required to form the open promoter complex. σ^N consists of three functional subunits (Merrick, Gibbins, and Toukdarian, 1987), region I which is highly conserved throughout RpoN homologues is required for the σ^N-holoenzyme to bind to the enhancer protein, but is not required for binding to the core RNA polymerase or the promoter consensus (Wong, Tintut, and Gralla, 1994;Cannon *et al.*, 1995). Region II is poorly conserved, but its loss in enteric bacteria decreases the rate of open complex formation (Cullen *et al.*, 1994). Determinants for binding to the core RNAP and DNA are located within region III, as well as playing a role in transcription initiation (Cannon *et al.*, 1994). Although σ^N plays an important role in a number of metabolic processes *in vitro*, it seems to be of little importance for virulence of *S.* Typhimurium via oral or IP infection (GR-unpublished observation).

1.4.6 The Phage Shock Response

The infection of a number of bacteria, including *E. coli*, *S.* Typhimurium and *Yersinia Enterocolitica*, with filamentous bacteriophage induces the phage shock response and specifically the phage shock protein (*psp*) locus (Brissette *et al.*, 1990) (Brissette *et al.*, 1990). Induction of the *psp* locus of *E. coli* is caused by the synthesis of a single filamentous phage protein, protein IV, a member of the secretin family of proteins (Possot *et al.*, 1992). Although named the phage shock response other inducers of the *psp* locus have been identified which include other secretins, mutant porins defective in membrane integration, proton ionophores, extreme heat shock, osmotic shock, sub-lethal concentrations of ethanol and lipid depletion (Bosch and Tommassen, 1987;Brissette *et al.*, 1991;Weiner and Model, 1994;Bergler *et al.*, 1994). This suggests a role for the Psp proteins in response to stress associated with membrane perturbation.

Thus far the only regulator of the *psp* locus outwith the locus itself is RpoN (Brissette *et al.*, 1991). All of the conditions that induce the phage shock response, other than secretin IV, induce the cytoplasmic and extracytoplasmic stress responses, but the phage shock response of *E. coli* can still occur in the absence of σ^H (Brissette *et al.*, 1991) or σ^E (Model, Jovanovic, and Dworkin, 1997). Positive regulation of the *psp* locus is dependent on the NtrC-like transcriptional activator, PspF (Jovanovic, Weiner, and Model, 1996), which is divergently transcribed to the rest of the genes which are situated in an operon, whilst the negative regulator of the operon is PspA (Brissette *et al.*, 1991;Weiner, Brissette, and Model, 1991;Weiner *et al.*, 1995). Interestingly *pspA* is induced in stationary phase, suggesting that the *psp* locus is switched off under this condition (Weiner and Model, 1994). A homologue of the *E. coli pspC* gene was shown to be important for virulence of *Yersinia enterocolitica*, whilst a *pspA* homologue was important for growth when the type three secretion system was active, and especially when the YscC secretin was being produced (Darwin and Miller, 2001).

1.4.7 The Extracytoplasmic Stress Response (ESR)

The response to stress which result in alterations to the composition or fluidity of the outer membrane resulting in a build up of outer membrane proteins within the periplasmic compartment is called the extracytoplasmic or periplasmic stress response. In the majority of Gram negative bacteria the ESR is governed by three pathways, the σ^E regulated pathway, the CpxAR two component pathway, and the BaeSR two component pathway.

1.4.7.1 The Alternative Sigma Factor, σ^E.

1.4.7.1.1 A Brief History of σ^E

Originally, the σ^E subunit of *E. coli* was purified from RNA polymerase derived from cells cultured at 50°C (Erickson and Gross, 1989) through its ability to initiate transcription of another sigma factor (σ^H) from the *rpoHP3* promoter. This was known to be the sole transcription site used for expression of *rpoH* under extreme temperature (Hiratsu *et al.*, 1995). They also identified that *htrA*, a gene that at the time had recently been shown to be essential for growth and survival at high temperatures in *E. coli* (Lipinska, Sharma, and Georgopoulos, 1988), possessed a σ^E regulated promoter. σ^E was also isolated as a contaminant in a RNA polymerase preparation that was relatively heat resistant (Wang and Kaguni, 1989) and again found to regulate transcription from *rpoHP3*.

The extracytoplasmic function (ECF) of sigma E (σ^E) was realised when expression of *rpoE* was observed to be induced by an increase of misfolded outer membrane proteins (OMPs) within the periplasmic space and was reduced when OMPs are removed (Raina, Missiakas, and Georgopoulos, 1995;Rouviere *et al.*, 1995). Activation conditions include oxidative stress, heat and ethanol, but generally any condition that leads to envelope distress. In addition, mutations in genes that result in an increase in misfolded OMPs also induce σ^E activity (Missiakas, Betton, and Raina, 1996). After screening several species (Lonetto *et al.*, 1994) proposed that a subfamily of eubacterial RNA polymerase factors regulated genes with ECF, one of which was σ^E. In *E. coli*, although initial work used null mutants (Raina, Missiakas, and Georgopoulos, 1995;Rouviere *et al.*, 1995), it is now accepted that in *E. coli* σ^E is an essential sigma factor for cell viability, with *rpoE* mutations proving lethal (De Las, Connolly, and Gross, 1997a). *rpoE* homologues are present in approximately a 100 species of bacteria. Interestingly null mutants in species closely related to *E. coli*, such as *S.* Typhimurium, *P. aeruginosa*, and others, are viable under normal physiological conditions but play

important roles in pathogenesis (Humphreys *et al.*, 1999;Hild *et al.*, 2000;Craig, Nobbs, and High, 2002;Kovacikova and Skorupski, 2002).

1.4.7.1.2 The σ^E Regulon According to *E. coli* K12.

Regulation of the σ^E pathway has been studied extensively in *E. coli* K12. Under non-stress conditions σ^E is inactive due to its sequestration to the cytoplasmic face of the inner membrane by its cognate anti-sigma factor RseA (Alba and Gross, 2004). Liberation of σ^E from RseA and activation of the σ^E pathway is dependent on a two stage cleavage of RseA by two proteases located in the inner membrane, DegS and YaeL (EcfE) (Alba and Gross, 2004). This pathway is depicted in figure 1A. DegS (HhoB) was first identified due to its homology to two periplasmic proteases, DegP (HtrA) and DegQ (HhoA) (Bass, Gu, and Christen, 1996;Waller and Sauer, 1996;Pallen and Wren, 1997). DegS possesses a membrane anchor, a serine protease domain and a PDZ domain (Alba *et al.*, 2001). In the absence of stress the PDZ domain of DegS inhibits the protease activity of DegS. The PDZ domain recognizes a YQF peptide sequence at the C terminal of outer membrane porins that accumulate in the periplasm during envelope stress or artificially by the overexpression of porins (Walsh *et al.*, 2003). This activates DegS protease activity leading to cleavage of the periplasmic domain of RseA (Walsh *et al.*, 2003). The second protease involved in σ^E activation, YaeL, is a human site-2 protease homologue, with 4 transmembrane domains (Kanehara, Ito, and Akiyama, 2002). YaeL acts on the cytoplasmic domain of RseA to liberate σ^E. Like DegS, the activity of YaeL is also negatively controlled by its PDZ domain (Kanehara, Ito, and Akiyama, 2003;Bohn, Collier, and Bouloc, 2004). In *E.*

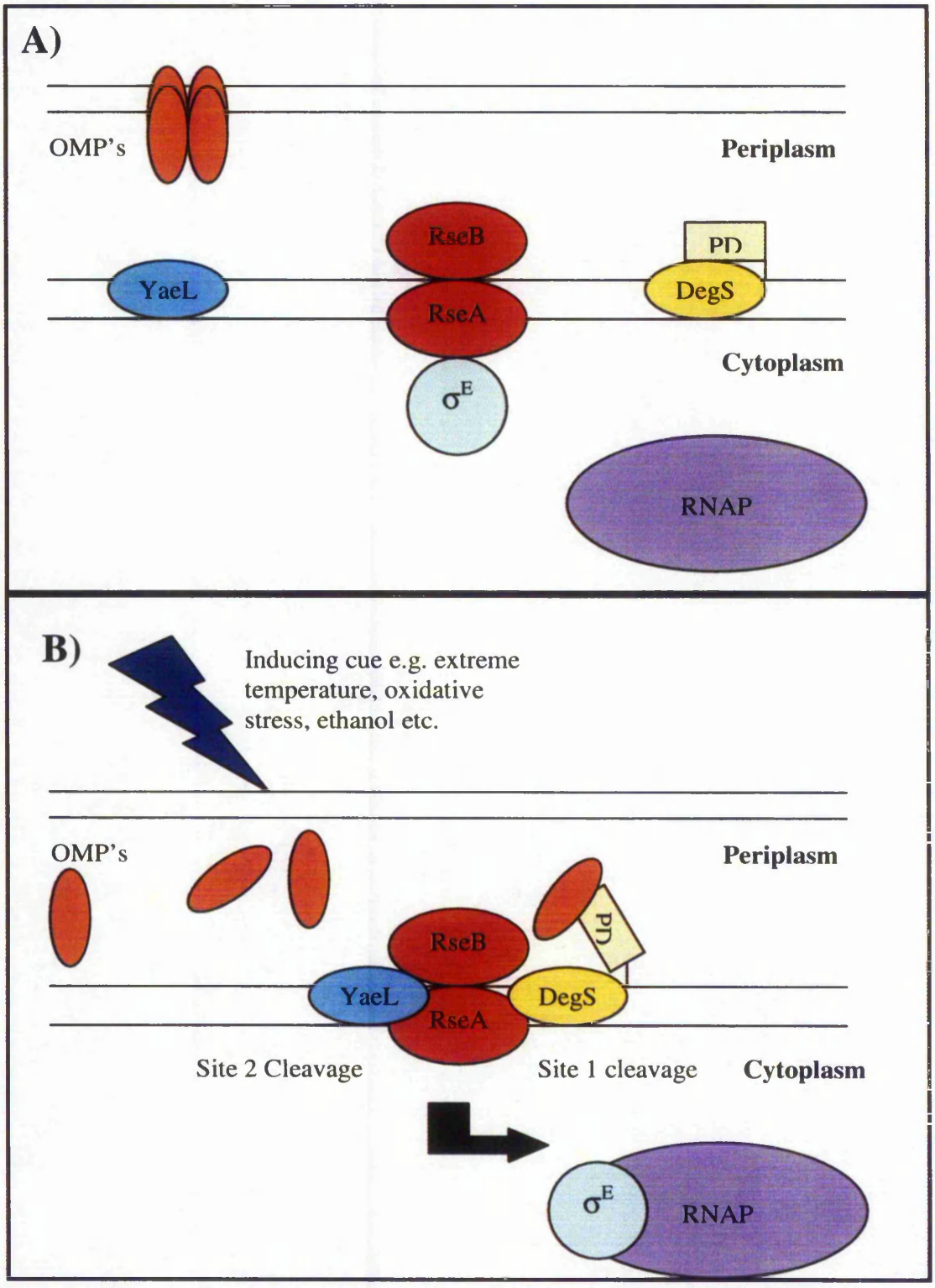


Figure 1A. Schematic Representation of σ^E Activation.

A) Non-activating conditions. Outer membrane porins (OMP) are inserted into the membrane in the correct orientation. The PDZ domain of DegS blocks the protease domain. σ^E is bound to the cognate anti-sigma factor RseA and cannot bind to RNAP.

B) Activating conditions. Misfolded OMP are located within the periplasm. These bind to the PDZ domain of DegS and activate protease activity. Two step cleavage of σ^E from RseA by DegS and then YaeL occurs. σ^E can bind to the RNAP and activate σ^E regulated genes.

coli, YaeL is not capable of cleaving RseA and liberating σ^E from RseA in the absence of DegS (Kanehara, Ito, and Akiyama, 2002;Alba *et al.*, 2002). Conversely the function of YaeL in *E. coli* has been suggested by others to negatively regulate the stress responses by degrading σ^E and another alternative sigma factor, σ^H (Dartigalongue, Loferer, and Raina, 2001). In *E. coli* the expression of YaeL, but not DegS, is dependent on σ^E (Dartigalongue, Missiakas, and Raina, 2001;Alba and Gross, 2004).

The search for σ^E regulated genes has been on going for a number of years, but with the advent of post genomic and proteomic techniques this search has been considerably aided. Up until this point the *E. coli* σ^E regulon consisted of itself, σ^H, HtrA (DegP) (Lipinska, Sharma, and Georgopoulos, 1988) and FkpA (Missiakas, Betton, and Raina, 1996), although other proteins had been implied but not confirmed. Dartigalongue, Missiakas, and Raina, 2001 characterised the *rpoE* regulon of *E. coli* using a combination of *lacZ* reporter genes as a screening method in various genetic backgrounds and 2D electrophoresis, and identified 20 σ^E dependent promoters, including those already mentioned. They used this as an explanation for the lethality of an *rpoE* mutant in *E. coli*. Following on from this (Rezuchova *et al.*, 2003) used a two plasmid method to try and identify σ^E regulated genes in *E. coli*. Here they identified 27 σ^E regulated promoters, from which a good consensus sequence could be derived, ggAACtt N_{16} TCnaA. Of these 27 promoters, 11 corresponded to promoters previously unreported as σ^E regulated, potentially regulating 15 genes. The use of different methodologies in characterisation of the *E. coli* σ^E regulon has identified a substantial number of differences in regulon members, indicating that no method is completely exhaustive for a task such as this. The genes described as σ^E regulated in *E. coli* are listed in Table 1. Some of these genes will now be discussed in more detail beginning with *htrA*.

Originally identified in *E. coli* through a mutant unable to grow at elevated temperatures (High Temperature Requirement) (Lipinska, Sharma, and Georgopoulos, 1988) and through the disability of a separate mutant strain to digest misfolded proteins within the periplasm (DegP) (Strauch, Johnson, and Beckwith, 1989). It is now recognised as a serine protease present in a diverse range of bacteria, implicated in a wide range of roles including tolerance of a wide range of stresses such as osmolarity, pH and oxidation, and also in higher eukaryotes (Gray *et al.*, 2000). In humans HtrA homologues have been proposed to have a functional role in arthritis and ageing (Gray *et al.*, 2000). A large degree of functional characterisation has now been completed on the *E. coli* variant and is known to form oligomeric complexes (Waller and Sauer, 1996) and consists of two PDZ domains in the C terminal portion involved in target recognition. At low temperatures *htrA* has general molecular chaperone activity and is able to stimulate refolding of chemically denatured substrates (Spiess, Beil, and Ehrmann, 1999). Proteolytic activity of HtrA is almost exclusively reserved for high temperatures and a temperature dependent switch from chaperone to protease has been defined (Spiess, Beil, and Ehrmann, 1999).

SurA was identified in a screen to identify proteins involved in the stationary phase survival of *E. coli* (Tormo, Almiron, and Kolter, 1990), and later as a novel peptidyl-prolyl-isomerase (PPiase) with homology to parvulin (Rahfeld *et al.*, 1994). Mutants of *surA* are more susceptible to bacitracin, vancomycin, and bile salts (Lazar and Kolter, 1996) and in this same study outer membrane proteins were observed which remained misfolded in its absence, therefore proving a role for *surA* in the maturation of these proteins.

1.4.7.1.3 The Role of σ^E in Pathogenesis

The importance of σ^E in pathogenesis became apparent through detailed analysis of it's orthologue in *P. aeruginosa*, AlgU (also called AlgT (DeVries and Ohman, 1994)). *E. coli* σ^E and *P. aeruginosa* AlgU are functionally equivalent (Yu, Schurr, and Deretic, 1995), with similar genetic organisation of the operon *algU mucA mucB mucC* (Deretic, Schurr, and Yu, 1995). Inactivation of *algU* decreases *P. aeruginosa* survival when exposed to certain external stimuli, including heat, and is less resistant to superoxide generators (Martin et al., 1994) (Schurr et al., 1995) (Schurr and Deretic, 1997) (Yu, Schurr, and Deretic, 1995). One of the major virulence factors of *P. aeruginosa* is alginate overproduction, and when overexpressed in the lungs of cystic fibrotic patients it is directly related to bacterial colonisation (Koch and Hoiby, 1993). Conversion of non mucoid strains to mucoid strains (alginate overproducing) occurs predominantly in strains possessing mutations in *mucA* (84% of strains analysed) (Martin et al., 1993), the anti-sigma factor of *algU* (Xie *et al.*, 1996;Schurr *et al.*, 1996;Mathee, McPherson, and Ohman, 1997). As is the case in *E. coli*, this leads to free AlgU within the cytoplasm which, when bound to core RNAP can drive expression from AlgU regulated promoters. One of these promoters is upstream of *algD* (Martin, Holloway, and Deretic, 1993) (Martin *et al.*, 1994), and it is increased expression of this gene that switches on the biosynthetic pathway of alginate, which results in a mucoid strain (Schurr *et al.*, 1993). *algD* is not solely regulated by AlgU, but also involves other regulatory elements, one of which is the transcriptional regulator *algR* (Wozniak and Ohman, 1994) (Deretic and Konyecsni, 1989;Deretic *et al.*, 1989;Kato *et al.*, 1989;Kimbara and Chakrabarty, 1989;Mohr *et al.*, 1992), itself also regulated by *algU* (Martin *et al.*, 1994). AlgU appears to be the most important of these however, as reversion from mucoid to non-

Gene Name	Proposed Function
rpoE	ECF sigma factor
rpoD	Housekeeping sigma factor
rpoH	Cytoplasmic function sigma factor
rseA, rseB, rseC	Regulatory genes of σ^E
skp	OMP assembly/folding
dsbC	Thiol:disulfide oxido-reductase
fkpA	Peptidyl prolyl isomerase
htrA	Serine protease (periplasmic)
surA	Peptidyl prolyl isomerase
yaeL	RIP protease
htrM	Lipopolysaccharide biosynthesis
lpxD	Lipid A (lipopolysaccharide precursor) biosynthesis
ecfA	Lipopolysaccharide biogenesis (speculated)
mdoG	Synthesis of membrane-derived oligosaccharide
cutC	Copper sensing
nlpB	Lipoprotein
yfiO	Putative lipoprotein (similarity to ComL)
yggN	Putative periplasmic protein
htrG	Putative IMP
yraP	Putative lipoprotein (similarity to OsmY)
yidQ	Putative IMP
ytfJ	Putative periplasmic protein
ecfK	Putative OMP (similarity to antigens Oma87, D-15-Ag)
yqjA	Putative IMP (DedA family)
lpxP	Cold shock induced palmitoleoyl transferase
psd	phosphatidylserine decarboxylase
sixA	HPt-specific phosphohistidine phosphatase
bacA	Undecaprenol kinase
sbmA	Transport inner membrane protein
smpA	Small outer membrane protein
yeaY	Outer membrane lipoprotein

ybaB	DNA repair
yiiS	Universal stress protein
yfeY	Unknown
fusA	Translation Elongation Factor

Table 1. A list of σ^E-regulated genes in *E. coli* determined by 2D protein electrophoresis (Dartigalongue, Missiakas, and Raina, 2001) and a two plasmid system (Rezuchova *et al.*, 2003).

mucoid has also been observed in strains harbouring spontaneous suppressor mutations of AlgU (Schurr *et al.*, 1994).

Another gene associated with alginate production, *algC*, is also regulated by AlgR. *algC* encodes for a bifunctional enzyme involved in lipopolysaccharide and alginate production (Coyne, Jr. *et al.*, 1994). The role of *algR* also includes regulation of type IV pili (Whitchurch *et al.*, 2002). An AlgR mutant is attenuated in a murine systemic model and has reduced ability to persist within the murine lung after aerosol inhalation (Lizewski, Lundberg, and Schurr, 2002). However a AlgU mutant demonstrates increased virulence in a *P. aeruginosa* mouse model of acute infection (Yu *et al.*, 1996), which was unexpected as strains with upregulated AlgU expression are often selected for during lung colonisation of cystic fibrotics. This may reflect the suitability of the mouse model used, as $1.8x10^6$ CFU of the wild type strain administered by the IP route did not result in a single mortality.

Using a promoter consensus search of the PAO1 genome (Firoved, Boucher, and Deretic, 2002) searched for members of the AlgU regulon, other than those involved in alginate production. They believed that alginate overproduction alone could not account for all of the clinical observations such as inflammation, seen upon conversion to mucoidy. They identified 10 novel putative AlgU dependent promoters. Two of these were upstream of lipoproteins, *lptA* and *lptB*. *lptB* has strong homology to the FKBP-like family of peptidyl-prolyl isomerases, and is known to induce IL-8 production in human macrophages. These findings strongly supported their argument that pro-inflammatory agents were co-induced with alginate production. This same group then used microarray analysis to compare gene expression in a mucoid and non-mucoid strain (Firoved and Deretic, 2003), to search for further members. This data confirms that AlgU regulated a number of pathways not involved in alginate production, which

could play important roles in the pathogenesis of mucoid strains in the lungs of cystic fibrotic patients. A similar study had also been performed with AlgR (Lizewski *et al.*, 2004). The extensive characterisation of the AlgU regulon involves some pioneering work for the role of σ^E homologues in pathogenesis, and has stimulated research into the role of σ^E in other pathogens.

1.4.7.1.4 The *Salmonella* Typhimurium σ^E Regulon

Due to the differences in both host and non host environments encountered by *S.* Typhimurium and *E. coli*, the conditions they have to adapt to are somewhat different (Winfield and Groisman, 2003). One might presume therefore that the ESR of an intracellular pathogen such as *S.* Typhimurium could be induced under different conditions to those described in *E. coli*, and subtleties in regulation and regulon members may have evolved due to exposure to these different environments. This is indeed the case for the σ^E regulon. In 1999, Humphreys et al, constructed a null mutant of *rpoE* in an otherwise wild type *S.* Typhimurium strain SL1344 and extensively characterised this mutant both *in vitro* and *in vivo*. The number of genes and the organization of the *rpoE* operons of *E. coli* and *S.* Typhimurium are identical. However, there are differences in the regulation and importance of σ^E to the two organisms in different environments. For example, σ^E is essential for the viability of laboratory strains of *E. coli* at all temperatures and members of the σ^E regulon are necessary for the growth of *E. coli* at 42°C, neither is the case for *S.* Typhimurium (Hiratsu *et al.*, 1995;De Las, Connolly, and Gross, 1997a;Humphreys *et al.*, 1999). When grown in L-broth the *S.* Typhimurium *rpoE* mutant does exhibit a protracted lag phase in the absence of glucose as a carbon source. It is significantly more sensitive to oxidative stress caused by hydrogen peroxide and paraquat and is more sensitive to the

antimicrobial peptide polymixin B than both wild type, SL1344 and a *htrA* mutant (Humphreys *et al.*, 1999). Probably the most important phenotype observed from this initial characterisation was the importance of σ^E for survival *in vivo*. The *rpoE* mutation significantly reduced the ability of *S.* Typhimurium to cause disease in BALB/c mice via both oral and parenteral routes of infection, with a LD50 some twenty times higher than that of the attenuated *htrA* (BRD915) mutant (Chatfield *et al.*, 1992c) (Johnson *et al.*, 1991). This indicated a role for other σ^E regulated genes in the pathogenesis of *S.* Typhimurium. The ability for an *rpoE* mutant to cause lethal infection in mice is however greatly increased in the absence of host superoxide generators such as NADPH oxidase, suggesting a function of σ^E is to resist oxygen dependent host defences (Testerman *et al.*, 2002). In agreement with the importance of σ^E in *S.* Typhimurium virulence, (Rollenhagen *et al.*, 2004) using GFP expression and two colour flow cytometry identified *rpoE* as one of the highest expressed genes *in vivo*.

σ^E also plays an essential role in the adaptation of *S.* Typhimurium to carbon starvation and stationary phase survival in a distinct but complementary role to RpoS (Kenyon *et al.*, 2002;Testerman *et al.*, 2002), although the Cpx arm of the ESR is not required for carbon starvation.

A predicted σ^E regulated gene, *fkpA*, had previously been shown to be important for survival of *S.* Typhimurium biotype Copenhagen (Horne *et al.*, 1997) in macrophage and epithelial cell types. However when the virulence of an *S.* Typhimurium SL1344 *fkpA* mutant was measured in a mouse model, there was no significant difference in numbers of the wild type, SL1344 and the *fkpA* mutant recovered (Humphreys *et al.*, 2003). Survival levels of this mutant in macrophage, Hep-2 and Caco-2 cells were also similar to that of wild type, SL1344, which was somewhat contradictory to results

previously published. A small defect could be seen if the strain also lacked *htrA* or *surA*, suggesting some compensatory function. The influence of *surA* mutations on the ability of *S.* Typhimurium to invade epithelial cells has also been investigated (Sydenham *et al.*, 2000). The invasiveness of a *surA* mutant in Hep-2 cells is 10 fold lower than that of the parent strain. In this same study the use of a *surA* mutant as a live vaccine was also considered. The *surA* mutant (BRD1115) has an LD_{50} some 4.5 log units higher than wild type, C5 strain, and is effective in immunising mice against wild type *S.* Typhimurium oral challenge. The *rpoE* mutant itself is a poor vaccine candidate as it is eliminated from the host too rapidly; however both *htrA* and *surA* are *rpoE* regulated genes which act as effective vaccine strains (Chatfield *et al.*, 1992c;Sydenham *et al.*, 2000). We aim to identify other σ^E regulated genes in *S.* Typhimurium and investigate their survival *in vivo*, in a move to identify future vaccine targets.

1.4.7.1.5 σ^E and Other Pathogens

Through the characterisation of σ^E in a variety of bacterial species a number of similarities and differences between the regulons have been reported, although the majority posses the same genetic arrangement. Like the *S.* Typhimurium homologue, σ^E is required for intracellular survival of non-typeable *Haemophilus influenzae* (NTHi) in murine macrophages (Craig, Nobbs, and High, 2002), and again an *rpoE* mutant is viable. Unlike in *E. coli*, σ^E of *Yersinia enterocolitica* is not induced by heat shock or ethanol (Heusipp, Schmidt, and Miller, 2003), although in this instance it still appears to be an essential sigma factor. However overexpression of the cognate anti-sigma factor, RseA, was not deleterious to growth, which might be expected if σ^E was essential for survival. The role for σ^E in two different species of *Vibrio* has also been elucidated (Hild *et al.*, 2000;Kovacikova and Skorupski, 2002), and is not essential for survival

under non-stress conditions for either. In the marine bacterium *Vibrio Angustum*, σ^E is induced by and required for survival at high temperatures. Interestingly in this case the downstream ORF does not have any similarity to any of the anti-sigma factors in the database, although it does code for an inner membrane protein with transmembrane regions, suggesting it may have an anti-sigma factor function. In *Vibrio cholerae* an *rpoE* mutant was analysed using an infant mouse model. In this model the *rpoE* mutant is defective for its ability to colonise and cause disease. Here again, the *rpoE* mutant was not induced by temperature shift or the addition of ethanol. Regulation of *rpoE* in *Vibrio cholerae* involves two promoters as it does in *E. coli*. P2 is σ^E dependent but the P1 promoter in *Vibrio cholerae* does not appear to be transcribed by σ^{70}.

1.4.7.2 The Two Component Regulator, CpxAR

As with *rpoE* the majority of the initial work performed on the Cpx envelope stress response was carried out in laboratory strains of *E. coli*. McEwen and Silverman first identified *cpxA* in 1980 via mutations that had an affect on the expression of the conjugative pilus (McEwen and Silverman, 1980). They observed that strains harbouring a mutant form of *cpxA* were no longer capable of forming a functional F pilus on the bacterial cell surface, and were therefore rendered incapable of conjugatively transferring the F plasmid. Other groups noticed that *cpxA* mutants had a variety of pleiotropic phenotypes all of which were linked to alterations in functions associated with the bacterial envelope (McEwen and Silverman, 1980;McEwen and Silverman, 1982;Silverman, 1982;Sutton *et al.*, 1982;Rainwater and Silverman, 1990). CpxAR is a two component regulatory system (Hoch and Silhavy, 1995) consisting of a sensor (*cpxA)* histidine kinase (HK) localised to the inner membrane by two transmembrane alpha helices that flank a single periplasmic domain (Albin, Weber, and

Silverman, 1986;Weber and Silverman, 1988) and a cytoplasmic response regulator (RR) (*cpxR)* (Dong *et al.*, 1993). *cpxA* like most histidine kinases acts as an autokinase, a RR (CpxR) kinase and a RR (CpxR) phosphatase (Weber and Silverman, 1988). The phosphorylation of CpxR enhances binding upstream of target genes (Pogliano *et al.*, 1997). The CpxAR two component regulator consists of another component other than the standard histidine kinase and response regulator. CpxP is a *cpx* regulated, small periplasmic protein which inhibits the regulon by binding to the sensing domain of CpxA, incorporating an extra level of regulation (Danese and Silhavy, 1998;Raivio *et al.*, 2000).

Envelope stress is transduced through the transfer of phosphate groups between CpxA and CpxR. *cpxA* null mutants possess an un-inducible phenotype, indicating that *cpxA* is essential for detection of envelope stress (Danese *et al.*, 1995), where as a deletion in the sensor domain of *cpxA*, results in a signal blind, constitutively active *cpxA* strain (*cpxA**). The initial role for CpxAR in the ESR was thought to be direct regulation of expression of genes encoding periplasmic proteins required for protein folding and degradation (Pogliano *et al.*, 1997). These include genes such as *dsbA*, *degP* and *ppiA* which are all upregulated in a CpxA* mutant (Danese *et al.*, 1995) or through overexpression of NlpE (Snyder *et al.*, 1995). A *cpxA** mutant shows numerous phenotypes including an anomalous positioning of the FtsZ ring in cell division (Pogliano *et al.*, 1998), decreased swarming ability, growth sensitivity to high temperature and an enhanced tolerance to amikacin and colicins A and K (De Wulf and Lin, 2000;Gubbins *et al.*, 2002). The physiological relevance of the *cpxA** phenotypes has been proven, with phenotypes demonstrated exclusively attributable to CpxR-P levels (De Wulf and Lin, 2000), although cross talk with another sensor was not eliminated.

Recent work on regulon members using promoter consensus searches and a CpxR-P recognition weight matrix (De Wulf *et al.*, 2002), implicates approximately 100 genes to have CpxR-P promoter boxes, these genes include those whose proteins have a role beyond the periplasm such as σ^H, a role in motility, MotABCheAW and Tsr, as well as regulation of its own operon. Interestingly a role for the negative regulation of the *rpoE* operon by CpxR-P was identified, and suggested that the Cpx response takes precedence over *rpoE* when confronted with two stress signals simultaneously. Using gene fusions and quartz crystal microbalance (QCM) (Otto and Silhavy, 2002) showed that an intact *cpxRA* signalling system and NlpE are required for productive cell surface interactions essential for stable adhesion.

The Cpx regulon has been implicated in the virulence of a number of bacterial pathogens (De Wulf, Akerley, and Lin, 2000). CpxA is involved in the pH-dependent regulation of *Shigella flexneri* VirF, a positive regulator of the *ipaBCD* invasion genes (Nakayama and Watanabe, 1998). DsbA is required for the folding and secretion of the IpaBCD proteins by *S. flexneri* and other virulence factors such as the enterotoxin produced by *Vibrio cholerae* (Yu, Webb, and Hirst, 1992;Yu, 1998). In *S.* Typhimurium Cpx regulates HilA a positive regulator of invasion genes (Nakayama *et al.*, 2003) and in *S.* Typhi using a TnphoA mutagenesis screen a *cpxA* mutant was found to have defects in adhesion and invasion of epithelial cells (Leclerc, Tartera, and Metcalf, 1998). CpxR has been described as a positive regulator of the *icm* and *dot* genes which are important for the infection process of *Legionella pneuomophila* (Gal-Mor and Segal, 2003). All of the above was deduced from *in vitro* assays. Recently our laboratory (Humphreys *et al.*, 2004) constructed strains of *S.* Typhimurium SL1344 with mutations in *cpxA* and *cpxR* rendering these genes inactive, as well as a constitutively active *cpxA** strain. The null mutants were not altered in their ability to attach to or invade

eukaryotic cells, however the *cpxA** mutant was significantly reduced in its ability to enter host cells due to an adhesion defect. In a murine model of systemic infection, virulence appeared to correlate inversely with levels of CpxR-P, with the *cpxR* mutant being the most virulent of the three strains and *cpxA** the least. This may suggest that CpxR-P negatively regulates genes that are important for infection, or positively regulate genes that interfere with murine infection.

1.4.7.3 The Two Component Regulator, *baeSR*

BaeSR (bacterial adaptive response) was first identified as a two component regulator in a library screen for two components on their ability to suppress *envZ* and *phoR/creC* mutants (Nagasawa, Ishige, and Mizuno, 1993). BaeSR is induced by similar conditions to those reported for *cpxRA* although overexpression of NlpE is not an inducing cue in this instance. Currently little is known about the regulon members although the periplasmic protein Spy, previously reported to be *cpxR* regulated is also regulated by BaeR (Raffa and Raivio, 2002). This adds an additional layer of regulation and complexity to the ESR of *E. coli* and its relatives. Another locus known to be regulated by this system is the multidrug resistant locus *yegMNOB* (*mdtABCD*) (Baranova and Nikaido, 2002). BaeR up-regulates this locus resulting in increased resistance to novobiocin and deoxycholate. In this same study *yicO* and *ygcL* were also identified as BaeR regulated although any further characterisation was not performed.

1.5 Treatment and Prevention of *Salmonella* Infection

1.5.1 Antibiotic Therapy

S. Typhimurium infections are normally self-limiting and there is no evidence in otherwise healthy patients that antibiotic treatment shortens the duration of symptoms. However a fraction of people infected with *S.* Typhimurium do go on to develop the

systemic form of the disease, for which antibiotic treatment is essential. For instance in children, the elderly or immunocompromised patients, *S.* Typhimurium infections are more frequently complicated by bacteraemia and systemic illness. In such cases the recommended treatment includes ciprofloxacin or trimethoprim (British National Formulary, 2004). Antibiotics are an essential part of the treatment regime for patients with systemic typhoid with ciprofloxacin, cefotaxime or chloramphenicol the current drugs of choice (British National Formulary, 2004). Antibiotics are also essential for treatment of carriers, but because the bacteria in carriers is localised to the liver and biliary tract, the antibiotic regime for clearance is different.

Multidrug resistant strains of both *S.* Typhimurium and S. Typhi are emerging as huge problems for the antibiotic treatment of infected individuals (Threlfall *et al.*, 1996;Rowe, Ward, and Threlfall, 1997). The problem of resistant strains of *S.* Typhimurium was first realised in the 1960s (Bulling, Stephan, and Sebek, 1973;Sojka and Hudson, 1976), and the first multi-resistant strain, PT 29 identified in 1964 with resistance to ampicillin, streptomycin, sulphonamides and tetracycline which caused several deaths in the vulnerable age groups (Anderson, 1968). From 1990 to 1995 the epidemic spread of multidrug resistant DT 104, with chromosomally encoded resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines has occurred (Threlfall *et al.*, 1996). Importantly since 1992 there has been an additional resistance to trimethoprim and a controversial decrease in sensitivity to ciprofloxacin, one of the front line drugs against *S.* Typhimurium infection (Threlfall, Ward, and Rowe, 1997;Threlfall *et al.*, 1997a;Threlfall *et al.*, 1997b). The consensus for the emergence of multidrug resistant strains of *S.* Typhimurium is the agricultural use of antibiotics which selects for antibiotic resistant strains of bacteria within the animals intestine, and DT 104 is no exception (Crerar, Nicholls, and Barton, 1999). The concern

with DT 104 is not just the multidrug resistance but also the predilection for this phage type to cause serious disease (Wall *et al.*, 1994). A 1998 outbreak of DT 104 in Denmark in a smoked pork product resulted in a number of systemic forms of *S.* Typhimurium infection which had to be treated with fluoroquinolones, although there were still two fatalities. (Molbak *et al.*, 1999)

Multiple antibiotic resistant strains of *S.* Typhi are now unfortunately relatively common amongst infections from the Indian subcontinent, the Middle East and South East Asia (Rowe, Ward, and Threlfall, 1997). The development of plasmid encoded resistance to chloramphenicol, ampicillin and trimethoprim is of major concern. Major epidemics of multi-resistant strains have been reported due to a contaminated water supply in Tajikistan (Murdoch *et al.*, 1998) and due to contaminated ice cream in the Philippines, discussed in (Threlfall, 2002). As mentioned, antimicrobial resistance in *S.* Typhimurium is thought to result from antibiotic use in agriculture whereas with *S.* Typhi and Paratyphi multidrug resistance is a direct consequence of antimicrobial usage in human medicine (Threlfall, 2002).

Due to the rapid increase in multidrug resistance in *Salmonella* species there is an urgent requirement to identify new bacterial targets for antimicrobial therapy. For an antibiotic to be a long term threat to a bacteria it must pose a problem to the bacteria that it has never encountered, and ideally will be a target that has never been exploited in nature, as the bacterial response will be slow as lateral gene transfer is less likely to be important. Hopefully the post genomic era will identify potential candidates.

1.5.2 Vaccines

Much of the current interest in *Salmonella* vaccines stems not only from their ability to immunise against *Salmonella* but also their potential to act as carriers of heterologous antigens, which will be discussed later. However the principal aim of developing

avirulent strains of *Salmonella* is to prevent *Salmonella* infections of humans and animals. Ideal vaccine strains should be genotypically stable and offer long term protection after one or two doses. However in practice the struggle in the development of such vaccines is to strike the balance between immunogenicity and reactogenicity (Everest, Griffiths, and Dougan, 1995).

Whole cell killed parenteral vaccines were not studied fully until the 1960s when the World Health Organisation commissioned several randomised, controlled field studies using two vaccines in typhoid endemic areas, although a preparation of heat killed *S.* Typhi and its efficacy against challenge with live counterparts had been attempted previously, reviewed by (Siler JF, 1941). In the WHO commissioned trials, one vaccine was heat-phenol inactivated, and the other an expensive and difficult to prepare acetone inactivated vaccine. The vaccines conferred good protection, up to 95% across some groups, but in a long term study of schoolchildren results were not as impressive (Tapa and Cvjetanovic, 1975). Both vaccines were responsible for severe side effects ranging from local injection site reactions to fever and malaise. These reactions seen with whole cell preparations are attributed to the reactogenicity of LPS (Hoops *et al.*, 1976).

There are currently two typhoid vaccines licensed for use worldwide, one parenterally administered and one orally (www.cdc.gov), although currently the oral vaccine is not available in the U.K.

1.5.2.1 Purified Vi Polysaccharide

The Vi capsule was first reported in the 1930s when (Felix A and Pitt R.M, 1934) observed that blood isolates of *S.* Typhi were expressing this capsule, and reviewed by (Robbins and Robbins, 1984) investigated its role as a protective antigen. The development of techniques that allowed large scale purification of Vi polysaccharide via

a non-denaturing method, led to a vaccine composed of the Vi capsular polysaccharide of *S.* Typhi, which has been licensed for use since 1990. A randomised trial using 25 micrograms elicited a significant rise in serum IgG Vi antibodies in ~90% adults and was well tolerated (Acharya *et al.*, 1987;Klugman *et al.*, 1996). When compared to the heat killed whole cell vaccine, the Vi vaccine is less reactogenic, effective in a single parenteral dose (although requires a boost every three years) and may be used in children as young as two years old. However, requirement of a boost every three years is not ideal for people living in endemic regions.

Vi behaves as a T cell independent antigen, but when conjugated to a carrier protein behaves as a T cell dependent antigen, giving rise to higher antibody levels and boostable memory (Szu *et al.*, 1987;Kossaczka *et al.*, 1999). Vi conjugate vaccines are still an area of ongoing research and field trials (Konadu *et al.*, 2000;Canh *et al.*, 2004).

1.5.2.2 Live Oral Ty21a

The first licensed oral typhoid vaccine consisted of a live attenuated strain of *S.* Typhi Ty21a (Germanier and Fuer E, 1975;Gilman *et al.*, 1977). The avirulence of this strain has been attributed to the mutation in *galE*, a gene in the galactose utilisation pathway that affects O antigen production and accounts for the rough LPS phenotype associated with this strain. However, the chemical method (nitrosoguanidine) used to mutate the parent Ty2 strain was likely to induce multiple mutations and Ty21a is now known to also possess several nutritional auxotrophies, mutations in the Vi capsular polysaccharide biosynthesis genes (*via)* (Silva *et al.*, 1987;Cryz and Furer, 1988), and in *rpoS* (Robbe-Saule, Coynault, and Norel, 1995) . A defined *galE via* mutant of *S.* Typhi is virulent in human volunteers (Hone *et al.*, 1988;Hone *et al.*, 1991). The nature of the Ty21a mutation is therefore obscure as is the possibility of reversion and whether the strain survives for long enough i*n vivo* to produce a good immune response is a concern.

As a vaccine Ty21a is only modestly immunogenic and requires three to four initial doses to elicit protection (Levine *et al.*, 1987;Levine *et al.*, 1996).

1.5.2.3 Live Oral Vaccine Candidates

Compliancy for vaccines against *S.* Typhi is still poor. In endemic regions this is often due to cost and availability. Oral vaccines are safer and easier to administer, avoid the associated risks of blood borne pathogens such as HIV in typhoid endemic regions due to re-use of needles, and are cheaper than the purified Vi vaccine which would hopefully increase there uptake in endemic regions. Oral vaccination with live attenuated *Salmonella* induces a serum IgG response as well as a secretory IgA (sIgA) response at mucosal surfaces (Staats *et al.*, 1994). Various investigators have undertaken to modify wild type *Salmonella* so that they are well tolerated but immunogenic enough so that one single oral dose elicits protective immunity. An aromatic amino-acid dependent *S.* Typhimurium *aroA* mutant strain was found to be avirulent in mice, and was presented as a potential vaccine strain by (Hoiseth and Stocker, 1981). The aromatic pathway was subsequently mutated in *S.* Typhi by introducing precise deletions in *aroC* and *aroD* resulting in strain CVD908 (Dougan *et al.*, 1988;Hone *et al.*, 1991). This strain is auxotrophic for aromatic amino acids and dependent on exogenous para-amino benzoic acid (PABA) for synthesis of folate and its availability is limited *in vivo* (Stocker, 1990). Phase 1 clinical trials demonstrate good tolerance and high levels of immunogenicity (Tacket *et al.*, 1992a;Tacket *et al.*, 1992b). A vaccine strain of particular relevance to this study is CVD 908-*htrA*, *htrA* being the σ^E regulated serine protease discussed earlier. This strain when administered orally produces an excellent immune response, but the silent vaccinemia seen with high doses of the parent CVD908 strain (Levine *et al.*, 1996), is no longer observed (Chatfield *et al.*, 1992b).

1.5.2.4 Delivery of Foreign Antigens

The use of attenuated strains of *Salmonella* to deliver foreign antigen evolved due to the relative success of attenuated strains of *S.* Typhimurium and *S.* Typhi to act safely and efficaciously as anti-*Salmonella* vaccines. Vaccine vectors are live carriers that act to deliver protective antigens from unrelated pathogens to the immune system of the vaccine recipient. Attenuated *Salmonella* strains make ideal vaccine vectors as they can be administered orally and induce both humoral and cellular responses (Curtiss, III *et al.*, 1989a;Curtiss, III *et al.*, 1989b). Also the ability to easily genetically manipulate *Salmonella* is useful in addressing the problems faced with this technique such as plasmid stability, copy number and the site of expression within the bacteria. The benefit of using *S.* Typhi to deliver heterologous antigen is that such vaccines may be used in developing countries, where immunisation against several diseases can be administered with a single dose. Antigens from a wide variety of bacteria, viruses and parasites, as well as eukaryotic proteins have been delivered to mice by attenuated *S.* Typhimurium and also using attenuated *S.* Typhi in human trials. Some disappointing studies using *S.* Typhi to carry hepatitis B antigens have been reported (Nardelli-Haefliger *et al.*, 1996), but successful studies using *Salmonella* strains expressing *P. falciparim* antigens (Gonzalez *et al.*, 1994) and fragment C of tetanus toxin (Fairweather *et al.*, 1990;Chatfield *et al.*, 1992a), where immunogenicity against the foreign antigen is evoked giving impetus to the possibility of using *Salmonella* as a vaccine vector.

1.6 Aims of Thesis

The degree of attenuation of an *S.* Typhimurium *rpoE* mutant, and the difference in attenuation between this and the σ^E regulated *htrA*, indicates a role for other σ^E regulated genes in the pathogenesis of *S.* Typhimurium. At the start of this investigation the *S.* Typhimurium *rpoE* regulon consisted of the *rpoE* operon itself, *htrA*, *fkpA* and *rpoH*.

The broad aim of this study was to identify putatively σ^E regulated genes of *S.* Typhimurium using a variety of methods, and compare the mutant phenotypes of these genes with those exhibited by the *rpoE* mutant, with the hypothesis that σ^E regulated genes may be used in further studies as possible vaccine strains or antimicrobial targets.

In order for this to be successful, rapid generation of chromosomal mutants was necessary and the primary aim of this study was to develop the λ Red mutagenesis system previously described in *E. coli*, for mutant construction in *S.* Typhimurium. After which, using a selection criteria, putative σ^E regulated genes were mutagenised and characterised. Secondly, regulation of the σ^E regulon has been extensively characterised in *E. coli*, but due to the differences in environmental niches we could expect that regulation of σ^E in *S.* Typhimurium could be different. Using promoter fusion studies we aimed to solve the regulation of the σ^E regulon of *S.* Typhimurium.

Chapter 2 - Materials and Methods

2.1 Bacterial Strains and Plasmids

The bacterial strains and plasmids used and constructed throughout this study are listed in tables 2 and 3 respectively.

2.2 Bacterial Media and Culture Conditions

All strains used in this study, unless for preparation of competent cells for lambda red mutagenesis, 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). All media was sterilised in an autoclave for 15 minutes at 121°C. Where required, media were supplemented with antibiotics at the following concentrations: 100μg/ml ampicillin (Sigma), 75ug/ml kanamycin (Invitrogen), 100μg/ml 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. Routinely, 5ml overnight cultures used for subculture were grown with aeration at 37°C in a rotary shaker (Stuart SI50). For production of inocula for infection studies, 200ml cultures were grown statically overnight in a 37°C incubator (Swallow), as were agar plates.

Pure cultures of all strains were maintained on microbank beads (ProLab) and stored at -80°C. When culturing from freezer stocks, one bead was removed and streaked onto a fresh LA plate containing the appropriate antibiotic.

Strain	Genotype	Source
***S.* Typhimurium**		
SL1344	*his* mutant mouse virulent strain	(Hoiseth and Stocker, 1981)
12023	mouse virulent strain	(Wray and Sojka, 1978)
GVB311	SL1344 *rpoE*::Kan	(Humphreys *et al.*, 1999)
GVB368	SL1344 *cpxR*::Kan	(Humphreys *et al.*, 2004)
GVB822	SL1344 *cpxA*::Gm	(Humphreys *et al.*, 2004)
GVB870	SL1344 *cpxA**	(Humphreys *et al.*, 2004)
SMS348	SL1344 *rpoS*::Amp	(O'Neal *et al.*, 1994)
GVB852	SL1344 *rpoN*::tet	SGSC
SMS561	SL1344 Δ*relA/spoT22*	M.P. Spector
BRD1115	C5 Δ*surA*	(Sydenham *et al.*, 2000)
GVB387	SL1344 *fkpA*::kan	(Humphreys *et al.*, 2003)
BRD915	SL1344 Δ*htrA*	(Chatfield *et al.*, 1992c)
GVB1322	12023 Δ*sbmA*::kan	This Study
GVB1333	12023 Δ*bacA*::kan	This Study
GVB1338	12023 Δ*yggN*::kan	This Study
GVB1336	12023 Δ*yggT*::kan	This Study
GVB1340	12023 Δ*degS*::kan	This Study
GVB1334	12023 Δ*stm1251*::kan	This Study
GVB1351	12023 Δ*oppD*::kan	This Study
GVB1339	12023 Δ*yiaD*::kan	This Study
GVB1341	12023 Δ*yccV*::kan	This Study

Strain	Genotype	Source
GVB1321	12023 Δ*tolR*::kan	This Study
GVB2011	12023 Δ*ptr*::kan	This Study
GVB2012	12023 Δ*rseC*::kan	This Study
GVB1361	12023 Δ*stm1250*::kan	This Study
GVB1307	12023 Δ*yraP*::kan	This Study
GVB1306	12023 Δ*stm1263*::kan	This Study
GVB1317	12023 Δ*yabI*::kan	This Study
GVB1310	12023 Δ*ybbN*::kan	This Study
GVB1308	12023 Δ*mlc*::kan	This Study
GVB1309	12023 Δ*dedD*::kan	This Study
GVB1319	12023 Δ*skp*::kan	This Study
GVB1316	12023 Δ*yrfH*::kan	This Study
GVB1342	SL1344 Δ*sbmA*::kan	This Study
GVB1348	SL1344 Δ*bacA*::kan	This Study
GVB1343	SL1344 Δ*htrA*::kan	This Study
GVB1370	SL1344 Δ*yggN*::kan	This Study
GVB1369	SL1344 Δ*yggT*::kan	This Study
GVB1361	SL1344 Δ*degS*::kan	This Study
GVB1346	SL1344 Δ*stm1251*::kan	This Study
GVB1347	SL1344 Δ*stm1254* ::kan	This Study
GVB1359	SL1344 Δ*oppD*::kan	This Study
GVB1371	SL1344 Δ*yiaD*::kan	This Study
GVB1358	SL1344 Δ*yccV*::kan	This Study

Strain	Genotype	Source
GVB1337	SL1344 Δ*ygiM*::kan	This Study
GVB1360	SL1344 Δ*tolR*::kan	This Study
GVB1305	SL1344 Δ*ptr*::kan	This Study
GVB1368	SL1344 Δ*rseC*::kan	This Study
GVB1363	SL1344 Δ*stm1250*::kan	This Study
GVB1365	SL1344 Δ*stm1250*::kan Δ*stm1251*::kan	This Study
GVB1372	SL1344 Δ*yraP*::kan	This Study
GVB 1377	SL1344 Δstm1263::kan	This Study
GVB1376	SL1344 Δ*yabI*::kan	This Study
GVB1380	SL1344 Δ*ybbN*::kan	This Study
GVB1344	SL1344 Δ*sbmA*::kan, Δ*htrA*	This Study
GVB1373	SL1344 Δ*mlc*::kan	This Study
GVB1357	SL1344 Δ*dedD*::kan	This Study
GVB 1367	SL1344 Δ*skp*::kan	This Study
GVB1374	SL1344 Δ*yrfH*::kan	This Study
GVB1398	SL1344 Δ*yaiW*::kan	This Study
GVB1381	SL1344 Δ*yggN*::kan, Δ*htrA*	This Study
GVB1375	SL1344 Δ*skp*::kan Δ*htrA*	This Study
GVB2021	SL1344 Δ*skp*, *fkpA*::kan	This Study
GVB1364	SL1344 Δ*stm1250*	This Study

Table 2 – A list of bacterial strains used and constructed throughout this study.

Name	Relevant Characteristics	Source
pKD46	pINT-ts derivative containing araC-P_{araB} and $\gamma\beta exo$ DNA fragments, Ap^R	(Datsenko and Wanner, 2000)
pKD4	pANT-Sγderivative containing an FRT-flanked Kan^R gene, Ap^R	(Datsenko and Wanner, 2000)
pKD3	pANT-Sγderivative containing an FRT-flanked Cm^R gene, Ap^R	(Datsenko and Wanner, 2000)
pCP20	bla cat cI857 lPR flp pSC101 oriTS	(Cherepanov and Wackernagel, 1995)
pTL61t	Low copy, promoterless *lacZ* fusion vector Ap^R	(Linn and St Pierre, 1990)
PCR 2.1	PCR topisomerase cloning vector	Invitrogen
pAC7	Low copy P_{BAD} (arabinose inducible) expression vector, Cm^R	(Rezuchova and Kormanec, 2001)
pAC-rpoEST4	pAC7 containing *rpoE* under control of P_{BAD}	(Miticka *et al.*, 2003)
pWSK29	Ap^r; low copy number vector.	(Wang and Kushner, 1991)
p*sbmA*	*sbmA* and promoter region in pWSK29.	This Study
prpoEP1::lacZ	*rpoEP1* promoter in pTL61t	This Study
prpoEP2::lacZ	*rpoEP2* promoter in pTL61t	This Study
propEP3::lacZ	*rpoEP3* promoter in pTL61t	This Study
pcpxRP::lacZ	*cpxRA* promoter in pTL61t	This Study
prpoHpall::lacZ	*rpoH* promoter region in pTL61t	This Study
pfkpaP::lacZ	*fkpaP* promoter in pTL61t	This Study

Name	Relevant Characteristics	Source
psbmaP::lacZ	*sbmaP* promoter in pTL61t	This Study
pSTM1263::lacZ	*stm1263* promoter in pTL61t	This Study
pSTM1251::lacZ	*stm1251* promoter in pTL61t	This Study
pyggtP::lacZ	*yggT* promoter in pTL61t	This Study
pyggNP::lacZ	*yggN* promoter in pTL61t	This Study
pyaeLP::lacZ	*yaeL* promoter in pTL61t	This Study

Table 3 – A list of plasmids used and constructed throughout this study

2.3 DNA Isolation

2.3.1 Isolation of Bacterial Chromosomal/Plasmid DNA

For rapid isolation of chromosomal and plasmid DNA to be used as template DNA in polymerase chain reactions (PCR), a boilate method was followed. A single colony was picked from a fresh plate and emulsed in 200µl sterile dH_2O. After boiling for 5 minutes at 100°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 Plasmid DNA

A QiaPrep minprep kit (Qiagen) was used for isolation of purified plasmid DNA employed as vector DNA in recombinant techniques, for electroporation or in PCR amplification of plasmid specific genes. Manufacturers instructions supplied with the kit were followed with the following exceptions. 5mls of an overnight culture were used as starting material, as opposed to the 2-3mls suggested, and plasmid DNA was eluted from the column using 50µl dH_2O rather than the EB buffer supplied.

2.4. Transfer of DNA

2.4.1 Electroporation of Bacterial Cells

For routine transformation of plasmid DNA, or for ligation reactions requiring a high level of transformation efficiency, electrocompetent cells were prepared. 5ml overnight cultures of the desired host strain were cultured in LB broth at 37°C. The overnight was then diluted 1/100, usually 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 benchtop centrifuge (Jouan CR3) at 5000rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 30mls ice cold 10% (v/v) glycerol and centrifuged as above. This washing procedure was repeated three times 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 a 100^{th} of the initial culture volume of ice cold 10% glycerol (i.e.) 500µl of cells would be produced from a 50ml starter culture. Cells were stored on ice or at -80°C until required for electroporation. 3µl of plasmid DNA were 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, 25µF 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) (Sambrook, Fritsch, and Maniatis, 1989) was added to the bacteria which were allowed to recover at 37°C for 1 hour. Aliquots of cells (usually 50 and 100µl) were plated out on LA plates containing the appropriate antibiotic for selection. A "no DNA" negative control was set up for each experiment.

2.4.2 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°C for 30 seconds. Immediately after shock, 250µl 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.

2.4.3 P22 Phage Mediated Transduction of *S.* Typhimurium.

P22 transduction was used to enable movement of gene mutations marked with antibiotic selection markers between strains of *S.* Typhimurium. This procedure was performed using the bacteriophage P22 and the method was adapted from that described by (Miller, 1972). Dilutions of the P22 stock were made in LBEDO broth, 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, which is poured over the surface of a dry LA plate containing appropriate antibiotics. After incubation for approximately 6 hours, or until plaques are visible, plaques are harvested from the plate which is just confluent. This is done by scraping off the top agar into a 50ml centrifuge tube with the addition of 3mls LBEDO and 4 drops of chloroform from a pastette, which is then incubated at 37°C for 30 minutes with shaking. This mixture is then centrifuged at 5000rpm for 10 minutes, the supernatant from which is removed and filtered through a 0.2µm filter syringe. More chloroform (4 pastette drops) is 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 desired transduction.

2.5 Molecular Cloning Techniques

2.5.1 Topisomerase Cloning

This method utilised the PCR2.1 kit (Invitrogen) which allows direct cloning of a *taq* polymerase PCR product (or a 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 allows blue/white screening on LA plates containing 50µg/ml of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside, with white colonies containing the desired insertion due to disruption of the vector *lacZ* gene.

2.5.2 Restriction Endonuclease Digestion of DNA

Restriction endonucleases were supplied by Invitrogen except for *dpnI* supplied by Promega. Digestion of all DNA was performed in a standard digest mixture composed of 1X the appropriate restriction digest buffer, 0.5-2µg 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°C waterbath (Grant) for at least 3 hours.

2.5.3 Ligation of DNA

Insert DNA was generally produced 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

observed on an agarose gel, with concentration depending on product size. For ligation reactions, both vector and insert DNA were ethanol precipitated post digest using pellet paint (Novagen). The resulting pellets were resuspended in 10µl of dH_2O and 1µl observed by agarose gel electrophoresis to quantify DNA concentrations. 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°C. A negative control ligation reaction for each digested vector used was performed to measure levels of vector re-ligation.

2.5.4 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 of purified mini-prep plasmid DNA.

2.5.5 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 run at 100V for up to 1 hour depending on gel length. 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).

2.5.6 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, of 5' and 3' oligonucleotides, 2µl of template DNA and 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 a initial denaturation at 95°C;2mins followed by 30 cycles of 94°C;40secs, anneal;30secs and a 72°C extension, with a final extension period of 72°C;10mins. Oligonucleotides for the purpose of generating insert for cloning and for screening were synthesised by MWG Biotech. All oligonucleotides used in this study are listed in table 4.

2.6. Nucleotide Sequencing

For sequence verification of the promoters cloned into pTL61t nucleotide sequencing was performed using a capillary sequencer (ABI 3100) and a big dye terminator V3.0 sequencing mix (ABI prism). Briefly, 200-500ng of plasmid DNA, 3.2 pmol of sequencing primer and 6µl of the big dye mix were combined in a 20µl reaction. Incorporation of the fluorescent dyes into the DNA were performed on a hybaid PCR express machine for 25 cycles at 96°C;30secs, 50°C;15 secs and 60°C;4 mins. PCR reactions were concentrated and purified using NF pellet paint (Novagen) and resuspended in 25µl HiDi formamide. Typically ~800bp of good sequence is achieved by this method.

Oligonuclotide	Sequence 5' to 3'
HisCMRedF	gcgctggacggtaaacgtatcgctacctcatatccgcacctccatatgaatatc ctccttag
HisCMRedR	cgatttaaaagagacgcctttctggtcgaggtagcgtttgagtgtaggctgga gctgcttcg
HisExtF	atgttagacaacacccgctta
HisExtR	tcactccatcatcttctcgat
SkpRedF	tttattgtgaaaaagtggttattagctgcaggtcttggtttggcgatggtgtgtag gctggagctgcttc
SkpRedR	cattacttatttaacctgtttcagtacgtcagcggtgatgtctttcacatcatatga atatcctccttag
Stm1251RedF	ttaatgatggcactcagaaccttgtcagcacttcccgtgtttgctgattcgtgtag gctggagctgcttc
Stm1251RedR	acgcgattatgatttgtgttcaatcgcctttggtttgctttctatggcaacatatga atatcctccttag
HtrARedF	aaacacatgaaaaaaaccacattagcaatgagtgcactggctctgagtttgtgt aggctggagctgcttc
HtrARedR	aggtgattactgcatcagcaaataaatagaactatcaccacgctgaatatcata tgaatatcctccttag
YaeLRedF	ggttttatgctgagtattctctggaatctggctgcattcatcatcgcactgtgtag gctggaagctgcttc
YaeLRedR	actctcttacaaccgagagaaatcattgaaagtgcaagccccattaacacatat gaatatcctccttag
YbbNRedF	gacgccatgtccgtacagaacattgtcaatattaatgaatgtgtaggctggagc tgcttc
YbbNRedR	gagaggattaatacagtagcgcgtacaactggcggcggtacatatgaatatc ctccttag
PtrRedF	ccgtgaatgccccgcagcacctggttcaaagcgttattgtgtgtaggctggag ctgcttc
PtrRedR	gcgacatcattcattcttttcgctcattagaggtaacgttcatatgaatatcctcct tag
Stm1263RedF	cgttttggtgattcatttaaaaaaactgacaatgcttctggtgtaggctggagct gcttc
Stm1263RedR	atcagacttaatgatgtagcatttcgtcaacgacctcattcatatgaatatcctcc ttag
YfiORedF	aacgtcatgacgcgcatgaaatatctggtggcagcagccagtgtaggctgga gctgcttc
YfiORedR	attgcttcaggtgtttttgctgttagcggcaataattttccatatgaatatcctcctt ag
YaiWRedF	acgttatgtcggcggcgtcccgtctttatcctctgcctttgtgtaggctggagct gcttc
YaiwRedR	ttatcgccccatacaccgcgcccgccgctcatcaactcgtcatatgaatatcct ccttag

Oligonucleotide	Sequence 5' to 3'
TolRRedF	taagccatggccagaacgcgtggacgaggtcgtcgcgaacgtgtaggctggagctgctt
TolRRedR	cagacttcagattggctgcgtcattaagccaaccgattttcatatgaatatcctccttag
Stm1250RedF	catattttgttcctggtggtcttaccgccagttgctgaaagtgtaggctggagctgcttc
Stm1250RedR	tcccgttcattgccatttccgctgtcccttcatctttacacatatgaatatcctccttag
YraPRedF	gtacacatgaaggcattttcgccgctcgcagtccttatttgtgtaggctggagctgcttc
YraPRedR	acaccggttacttaatatacgtaaaggcggtggtcacgcgcatatgaatatcctccttag
YabIRedF	cacactatgcaagcactgctggaacactttatcacccaatgtgtaggctggagctgcttc
YabIRedR	gaatcgttagtaacccacaactttgcgcagaatatcgatacatatgaatatcctccttag
MlcRedF	gagcggtggttgctgatagtcagcctgggcatatcgatcagtgtaggctggagctgcttc
MlcRedR	cagttaaaaaatgttaaccctgtaatagacgaatcaacaacatatgaatatcctccttag
DedDRedF	gaatcggtggcgagtaagtttcagaatcgtttagtcggcagtgtaggctggagctgcttc
DedRedR	acgcgccttagttcgggctatatcccatcaccacgccgctcatatgaatatcctccttag
YrfHRedF	gacgccatgaaagaaaagtcctccgttgaggtcagactgggtgtaggctggagctgcttc
YrfHRedR	tgacagttattcgctgtcgccgtgtttaaatcgtaacaggcatatgaatatcctccttag
YccVRedF	gtgactatgatagccagcaaattcggtatcggccaacaggtccgccattcgtgtaggctggagctgcttc
YccVRedR	atacaatcagttacgtagtcgcggcgcctgaagctgcttgcgaatggtaccatatgaatatcctccttag
DegSRedF	tccatcatgtttgtgaagctcttacgttcggtcgcaataggtttaattgtgtgtaggctggagctgcttc
DegSRedR	gtcgttttagttcgacgccgggtattcctgcaccgtcacctggaacgtgacatatgaatatcctccttag
RseCRedF	agtacaatgattaaagagtgggcaaccgttgtctcctggcagaatggtcagtgtaggctggagctgcttc
RseCRedR	aaacgtttactggcgcgtttcgattgaggtagtttcgacgcgaacaaggtcatatgaatatcctccttag
RpoHRedF	atttgaatgaccaaagaaatgcaaaatttagctttagcccctgttggcaagtgtaggctggagctgcttc
RpoHRedR	agattacgcttcgatcgcagcgcgaagctttttcatggcgttcttttcaacatatgaatatcctccttag
SbmARedF	gagagcatgatgtttaaatctttttcccaaagccagggccgtttttcatgtgtaggctggagctgcttc

Oligonucleotide	Sequence 5' to 3'
SbmARedR	cttctgttagctaaaagagtgggtcacttcctgaactggctgaccatccacatat gaatatcctccttag
BacARedF	ggtttattgatgagcgatatgcactcgctgctgatagcggcaattttggggtgt aggctggagctgcttc
BacARedR	caatcatcagaagaagacaacgtaaacggcagccgccaccacaaagcggt catatgaatatcctccttag
YiaDRedF	tttaagatgaaaaaacgtgtatttgtgattgctgccatcgtgagcggcgcgtgt aggctgagctgcttc
YiaDRedR	ggtagtttactgcaatggactcaaggtaatttcgacgcggcggttttgcgcata tgaatatcctccttag
YggTRedF	aacgccatgaatacgttgaccttcctgctctcaacggtaattgagctgtagtgta ggctggagctgcttc
YggTRedR	cagcactcataacgccatccacagccccggcagcaacatgttgcctgtcgca tatgaatatcctccttag
YggNRedF	aaagttatgatgcgcaaaacgctgctggcgacagtgctaacgtttacggcgtg taggctggagctgcttc
YggNRedR	gaaagattacttcaggctgccaaccagcgctttgcggctatcttctaaggcata tgaatatcctccttag
oppDRedF	acagccatgagcttatcagaaaccgcaactcaggcgccgcaaccggcgaa gtgtaggctggagctgcttc
oppDRedR	cagcattcatagcagttcctccaccggtttaaaacaggcgcgcaagcgacca tatgaatatcctccttag
Stm1254RedF	aatcccatgacaaataaaaaacacatttttcaattatctttattggttcgtgtagg ctggagctgcttc
Stm1254RedR	gtgttgttattgtcctcttacgacaacacaggttttaccataaaacggatcatcat atgaatatcctccttag
YgiMRedF	agcctgatgccaaaattacgcctgattggactaactttacgtgtaggctggag ctgcttc
YgiMRedR	ggcgatttagttcatccagcggtctttgcgtttacggcttcatatgaatatcctc cttag
YcbKRedF	attatcatggacaaatttgacgctaatcgccgcaagctgcgtgtaggctgga gctgctc
YcbKRedR	cgctgattaccagtgccgcgccggcccggtatcaatatgccatatgaatatc ctccttag
SkpExtF	ggggccgttggtcttctcctacg
SkpExtR	ggattcaccataaacgtaatgtg
Stm1251ExtF	ttgccttgatgttgaacttt
Stm1251ExtR	cgctacagacaatgaaggcc
HtrAExtF	tgcgttacctgttaatcgag
HtrAExtR	ccatggcggaaggggggacaa

Oligonucleotide	Sequence 5' to 3'
YaeLExtF	tgttactggtgttcaggacg
YaeLExtR	catcgccatcgttattatgc
YbbNExtF	gcgaaagtacgtcatgcttt
YbbNExtR	gcgtcatcagttggtagtga
PtrExtF	gtctcgttgattgtatgaaa
PtrExtR	gcagtagccgcagatacagc
Stm1263ExtF	ctcaaccggatagagcatag
Stm1263ExtR	ctgtgacacgagtaatcagg
YfiOExtF	tgagttgtactcgttgtgcc
YfioExtR	ccgttcttaatgtcaaaatg
YaiWExtF	ttgttgccggtacgattacg
YaiwExtR	acaggtcgaccgcgggtaga
TolRExtF	gcacggtaggctctatcagt
TolRExtR	tcgatagcggaaccaccgcc
Stm1250ExtF	agacgatagctaaaattcca
Stm1250ExtR	tcctgttaattgactgaaaa
YraPExtF	gcagccgtcacacgtgacat
YraPExtR	gctattccggctgttgccag
YabIExtF	atcagcgtatcagccaggcg
YabIExtR	actgctgctggatgaaccgt
MlcExtF	tactggctgcccgacgggat
MlcExtR	agatatggcaagggcaatca
DedDExtF	gttgacgactggtattgtgc

Oligonucleotide	Sequence 5' to 3'
DedExtR	ctgccatcggcctgataagc
YrfHExtF	agcataccggtctggcctca
YrfHExtR	atgtcaccagcaaacttcag
YccVExtF	atgccgcaatagatgccggt
YccVExtR	catgaccgtggtgatggatc
DegSExtF	gcggcaacgagaacatttat
DegSExtR	ccagtctttattgactagtc
RseCExtF	agatgctgcgcaccgggcgc
RseCExtR	gtgtaaggagagcgggaact
RpoHExtF	ggtgactgaagtggcgcaggtctt
RpoHExtR	ggaatagctaaatttacatccaga
SbmAExtF	tacgaaacacgcgttgattgccgtc
SbmAExtR	ctcctgctgggcgacagccagcacc
BacAExtF	atgctcagtccgcagcttga
BacAExtR	ggtgtttacgactgggaaca
YiaDExtF	gccacggtcgctgtggtaaa
YiaDExtR	ccgggtaaagcatgccgcct
YggTExtF	atcccggctccagagtcaga
YggTExtR	ggtaatggcgattttcacct
YggNExtF	gcctgggaagggctgatttg
YggNExtR	aaccagcgatatatatccga
oppDExtF	atggcctgcgtgatgccctc
oppDExtR	cactgcttcccttctttgat

Oligonucleotide	Sequence 5' to 3'
Stm1254ExtF	agatagcggcacgtaaaatg
Stm1254ExtR	gacggtaagttttctattaa
YgiMExtF	gatagtattttgcgccaaat
YgiMExtR	tcctctaatgataagacagt
YcbKExtF	tgacgcgggctttatcggca
YcbKExtR	tgcagccagaactcatcttc
Stm1250ExtRNew	agtcacgaatttaagtggtt
pTL61tF	gaattctcatgtttgacagc
pTL61tR	ggtcatagctgtttcctgtg
rpoEP1F	cccaagcttgtcggattttgtagt
rpoEP1R	gctctagacgtttgcagagtaaccta
rpoEP2F	cccaagcttgtacagatagtgcgt
rpoEP2R	gctctagacttatcttccattataag
rpoEP3F	cccaagcttataatgatagataatgatccgtct
rpoEP3R	gctctagaccaaatttccacgcgctatcgaaa
cpxRPF	cccaagcttaaaactgaatgccagcgttga
cpxRPR	gctctagatgtttacgtacctccgaggcag
rpoHpallF	cccaagcttattggctggatcgccgcc
rpoHpallR	gctctagatcagtattgcctggcacc
fkpaPF	gctctagaccatatctccagggccggggc
fkpAPR	cccaagcttgctttcacagcaacattacag
stm1263PF	cccaagcttgggttagctcaaccggat
stm1263PR	gctctagagccaccaaaacgttacc

Oligonucleotide	Sequence 5' to 3'
yggNPF	cccaagcttggggtcttctatcgacgggtaca
yggNPR	gctctagagcttagtcccaccaaa
yggTPF	gctctagagcaaacgatgtaaagcc
yggTPR	cccaagcttggggtggaaggcctgcac
stm1251PF	cccaagcttggggcccgaaggcggcgctgggtt
stm1251PR	gctctagagccattaacctcctgaatcacta
yaeLPF	cccaagcttggggcaagggtttatcggcgggct
yaeLPR	gctctagagcaaaaccttccgttagagcgtc
sbmaPF	cccaagcttgggcagaaatgcgaacta
sbmAPR	gctgtagagcaaagggctaaaccag

Table 4 – A list of oligonucleotides designed and used throughout this study.

2.7. SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the standard method described by Laemmli (Laemmli, 1970). Ready-made 12% acrylamide gels were used (BioRad). Whole cell protein samples were prepared from bacterial cultures at the desired OD600nm. Sample aliquots were 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°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 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 was added in 5% Marvel PBS-tween and left to shake on an orbital shaker for at least 2 hours. Unbound antibody was removed during three 15 minute washes with PBS-Tween. 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 100µl 30% H_2O_2) were added to the blot and left until colour developed and protein bands could be visualised.

2.8. 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 (Miller, 1972), but adapted into a 96 well microtitre plate format (Griffith and Wolf, Jr., 2002). 5ml overnight cultures were grown with ampicillin to select for the reporter plasmid, and diluted 1/100 into 25ml fresh LB broth. β-galactosidase activity was measured every hour during a growth curve or at a specific OD600nm. Seven individual 100µl aliquots of bacterial cells per strain were tested. Bacterial cells were lysed with 1 pastette drop of 1% SDS and 2 pastette drops of chloroform and vortexed for 10 seconds. After 5 minutes 10µl of the suspension was added to 90ul of assay buffer (16.1g Na_2HPO_4, 5.5g NaH_2PO_4, 0.75g KCl, 0.24g $MgSO_4.7H_20$, 2.7ml β-mercaptoethanol per litre). 25µl of 4mg/ml ONPG (o-nitrophenol-β-D-galactopyranoside) in assay buffer without β-mercaptoethanol, was then added to the reaction which acts as a colour substrate to the enzyme. The time taken for a yellow colour similar to that of LB broth to develop was accurately recorded and the reaction stopped with 50µl 1M Na_2CO_3. The final OD420nm and OD550nm were then recorded and promoter activity measured using the following calculation:

Promoter Activity in Miller Units =

$$\frac{1000 \times (OD420nm - (1.75 \times OD550nm))}{\text{time (minutes)} \times \text{cell volume (mls)} \times OD600nm}$$

For *S.* Typhimurium strains carrying both a reporter plasmid and plasmid pAC-*rpoE*ST4, which were constructed to compare the effects of overexpression of σ^E on

promoter transcription, the above method was adapted slightly to one similar to that described by (Chen *et al.*, 1995). Overnight cultures of strains containing the appropriate plasmids were diluted 1/1000 into fresh LB broth with the addition of antibiotics. Cultures were allowed to grow to early log phase. Bacteria were collected by centrifugation and diluted to an OD600nm of approximately 0.05 in pre-warmed LB broth containing 0.2% arabinose (pBAD inducing culture) or 0.2% glucose (pBAD inhibitor control). Samples were taken every hour and assayed for β-galactosidase activity in the same way as described above.

Promoter-*lacZ* fusion activity was also measured from *S.* Typhimurium carrying reporter plasmids post invasion of the murine macrophage cell line, RAW 264.7. The invasion assay was carried out as described by (Everest *et al.*, 1995). After macrophage cell lysis the cell lysate was treated as a bacterial culture above and assayed for β-galactosidase activity. Viable counts of the lysates were made and extrapolated to a standard curve of *S.* Typhimurium to calculate the equivalent OD600nm.

2.9 Growth Phase Kinetics

The ability of mutant strains of *S.* Typhimurium to grow in liquid culture was compared with the wild type parent strain. A number of growth curves were performed for each mutant constructed in this study under a number of different environmental conditions. This was done in two ways:

1) Overnight cultures were diluted 1/100 into fresh LB broth in 50ml conical flasks and grown aerobically at the desired temperature to be tested with OD600nm measured every hour on a spectrophotometer.

2) Overnight cultures were diluted 1/100 from which 200µl were aliquoted into a 100 well plate (Thermo-hybaid) and grown aerobically with shaking in a Bioscreen C

machine set at the desired temperature. The Bioscreen C machine then records the OD600nm at the desired interval. Growth curves obtained from both of these methods appear to be equivocal.

2.10 Disc Diffusion Assay

Bacteria were cultured overnight in 5ml L-broth containing the appropriate antibiotic. Overnight cultures were diluted 1/10 into fresh L-broth and incubated with shaking for 1 hour at 37°C. 100µl of bacteria were added to 3mls of Top agar, and poured onto dry L-agar plates to form a bacterial lawn. For hydrogen peroxide sensitivity, 6mm paper discs (Whatman, UK) were placed onto the plate and 10µl of 3% H_2O_2 added per disc. For Polymixin B sensitivity, discs containing 300U Polymixin B (Oxoid, UK) were added. Polymixin B is an antimicrobial peptide which binds to the lipid A core of lipopolysaccharide (LPS). After this binding the inner and outer membranes are permeabilised, which results in cell death. Plates were incubated at 37°C overnight and zones of inhibition measured. Sixteen replicates for each agent were completed per strain. Significance was measured using ANOVA with a Tukey-Kramer post test for comparison of more than two strains, or a student t test was used for comparison of just two strains.

2.11 Macrophage Invasion Assay

The murine macrophage cell line, Raw 264.7, was routinely cultured in MEM Earles media without L-glutamine (Gibco) supplemented with 2mM glutamine, 10% (v/v) foetal calf serum and non-essential amino acids. For invasion assays macrophages were seeded in 12 well tissue culture plates (Corning Costar) at a density of $1x10^6$ cells per well and incubated at 37°C with 5% CO_2 overnight. Prior to infection, cells were washed twice with 1ml of sterile phosphate buffered saline (PBS). 5ml overnight

cultures of bacterial strains to be investigated were grown in L-broth with the appropriate antibiotic and diluted into the above tissue culture media. 1ml of media containing $1x10^6$ bacteria was added to the monolayers to achieve a multiplicity of infection (MOI) of ~1:1. Infected cells were incubated for 2 hours and washed three times with 1ml of sterile PBS, after which tissue culture media containing 100μg/ml gentamicin was added and incubated for a further hour. At this point cells were observed microscopically to confirm gentamicin killing. For the three hour post infection time point macrophages were washed 3 times with sterile PBS and lysed with 1ml of ice cold water. The numbers of bacteria present were quantified via serial dilution onto L-agar plates. For the 24 hour time point, tissue culture media containing 10μg/ml gentamicin was added to the monolayers and incubated for a further 21 hours, where upon they were washed and lysed as per the 3 hour time point.

2.12 Mouse Infection Models

The majority of mice used in this study were female BALB/c between 6-8 weeks old (Harlan, UK). However to compare the virulence of some mutant strains in *ity*^{S} to *ity*^{R} mice strains, female CBA and DBA/2 mice were used of a similar age, or for comparison in a more wild type strain, female MFI mice were used. At the experiment end point or when mice were considered too sick for the experiment to continue, mice were euthanized by cervical dislocation. For IP and IV routes of infection, 50mls of bacteria were cultured overnight without shaking whereas for oral infections 200mls of bacteria were cultured in the same way. The cells were recovered by centrifugation at 5000rpm for 10 minutes, washed once in 30mls sterile PBS, and resuspended in 5mls PBS. OD600nm of the dose were measured to estimate the CFU/ml and this was adjusted to obtain the desired dose per 200μl inocula.

2.12.1 Intraperitoneal (IP) and Intravenous Challenge

The virulence of wild type *S.* Typhimurium and mutant *S.* Typhimurium strains was compared using the well described competition index assay (Beuzon and Holden, 2001). A mixed inoculum was prepared usually containing approximately $1x10^3$ CFU of each strain per 200µl dose. Three to five female BALB/c mice (6-8 wks old, Harlan, UK) per group were infected via the intraperitoneal (IP) route or intravenously via the tail vein (IV), monitored daily and culled 3 days post infection. Livers and spleens were extracted and 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) is 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.

Single IP and IV infections were also performed with attenuated *S.* Typhimurium strains where measurement of CFU/organ was desired over periods longer than 3 days, which is possible in the absence of the wild type strain. Single infections were also used when the antibiotic markers of mutants were the same or where no antibiotic marker was present to differentiate it from the wild type strain.

2.12.2 Oral Challenge

To study virulence via the oral route, inocula were prepared as above. Only single strain infections were performed by this method. To investigate virulence of a strain, inocula containing approximately 5 x 10^7 CFU/ml were prepared, 200µl of which was administered via oral gavage. 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.

To measure the ability of a mutant strain to protect against *S.* Typhimurium infection, mice were inoculated as above and then challenged a month later with ~$1x10^{10}$ CFU/ml wild type *S.* Typhimurium, strain SL1344. Mice were monitored for up to six weeks and any deaths recorded. After six weeks mice were bled out and used for ELISA analysis of *Salmonella* specific antibodies.

2.13 Microarray Analysis

2.13.1 RNA Extraction

Overnight 5ml cultures of *S.* Typhimurium SL1344 harbouring pAC7 and SL1344 harbouring pAC-*rpoE*ST4 in LB-chloramphenicol were grown at 37°C with aeration. These cultures were diluted 1/100 into fresh LB-Cm containing 0.2% arabinose, and grown to an OD600nm of 0.5. At which point the Institute of Food Research (IFR) RNA extraction method (www.ifr.bbsrc.ac.uk), based on a SV total RNA kit (Promega), was followed. All solutions described below were commercially produced RNAse free solutions, and only used for the purpose of RNA extraction. 4mls (2.0 OD600 units) of each strain were harvested and transferred to a 50ml centrifuge tube containing 1/5 volume 5% (v/v) phenol and 95% (v/v) ethanol. This mixture was allowed to stand on ice for 30 minutes after which it was centrifuged at 3220xg for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in any residual liquid. This was transferred to a microcentrifuge tube and spun at the same conditions for 60 seconds. Remaining liquid was removed and pellets frozen at -80°C overnight. Next day, pellets were resuspended in 100µl TE buffer containing 50mg/ml lysozyme and incubated at room temperature for 5 minutes. The remainder of solutions used are constituents of the SV total RNA kit, and prepared as recommended in the manufacturers instructions. 75µl

of lysis reagent was added to the mix, mixed by inversion, and followed by addition of 350µl RNA dilution buffer, which again was mixed by inversion. Samples were heated at 70°C for 3 minutes and centrifuged at 15,000xg in a microcentrifuge for 10 minutes. Supernatants were transferred to a fresh RNAse free microcentrifuge tube with 200µl ethanol, mixed and transferred to a spin column, which was centrifuged at 15,000xg for 30 seconds. Eluate was discarded and the column washed with 600µl wash buffer, and re-centrifuged as previous. 50µl of DNase mix (5µl 90mM MnCl2, 40µl DNase core buffer, 5µl DNase) was added directly to the column matrix and incubated at room temperature for 15 minutes. This reaction was stopped with 200µl of stop buffer and centrifuged for 30 seconds. The column was serial washed with 600µl and 250µl wash buffer respectively with 30 second spins in between each wash. The column was then transferred to a fresh RNase free microcentrifuge tube and RNA eluted by the addition of 100µl RNase free distilled water which is allowed to stand for 60 seconds and centrifuged at 4500xg for 2 minutes.

Five samples were prepared per 25ml culture and the sample containing the highest quality RNA was used for microarray analysis. Each strain was repeated twice, to account for biological variation.

2.13.2 Quantification and Qualification of RNA

Integrity and quantity of RNA was assessed by spectrophotometer and by denaturing agarose electrophoresis. For spectrophotometric analysis 1µl of the RNA preparation is diluted 1/100 in TE (10mM Tris HCl pH 8, 1mM EDTA) and the A260 and A260/A280 ratios determined. RNA concentration was calculated on the basis that an A260 of 1 is equivalent to 40µg RNA/ml, and purity determined by an A260/A280 ratio of between 1.7 and 2.1. Typically each preparation produced 50-60µg of pure RNA.

2.13.3 RNA Labelling and Hybridisation

RNA labelling and hybridisation was carried out by Dr. Arthur Thompson, Institute of Food Research, Norwich onto *S.* Typhimurium LT2 and SL1344 arrays (see chapter 5).

2.14 Lambda Red Mutagenesis

The method described here is the culmination of experimental trials described in chapter 3, and is the most efficient protocol for this method in my hands.

2.14.1 DNA preparation

Using the desired template plasmid depending on the desired antibiotic resistance, 12 x 100µl PCR reactions were performed using Sigma Genosys oligonucleotides with 20bp homology to the antibiotic cassette, and 40bp homology to the chromosomal loci of interest. Due to the volume of the PCR reactions required a *taq* polymerase master mix was used as described in 2.5.4, 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.

After PCR the 1200µl product is concentrated and purified using Qiaquick PCR clean (Qiagen) kit, with 600µl aliquots passed through a single column. The DNA is eluted with 50µl ultra pure water per column. The DNA is then *dpnI* digested to remove template plasmid (2ul *dpnI*, 5µl reaction buffer per 50µl DNA), and then re-cleaned using another two Qiaquick PCR columns as above. This results in 100µl of purified DNA to use for mutagenesis.

2.14.2 Lambda Red Swap

Electrocompetent cells are produced as described in 2.4.1. 5mls of the desired parent strain carrying the temperature sensitive helper plasmid pKD46 are grown overnight at 30°C in LB-Amp, and then diluted 1/100 into 100mls fresh LB-Amp carrying 1mM L-arabinose, and cultured at 30°C with shaking until an OD600nm of 0.6 (usually around 4 hours). Cells are concentrated 100 fold so this starting volume provides 10x100µl aliquots. 100µl of electrocompetent cells are mixed with 0, 1, 5, 10, 20, or 30µls of purified DNA, and rested on ice for 30 minutes. After electroporation as per 2.4.1, cells are recovered for 2 hours at 37°C. 100µl of bacteria are plated out on LA plates carrying the antibiotic corresponding with the chosen template plasmid, and incubated at 37°C. If after 24 hours there are no colonies, the remaining 900µl stored at room temperature overnight, is centrifuged at 5000rpm for 2 minutes, and resuspended in 100µl SOC, plated out and cultured as above. Mutagenesis is confirmed using primers designed externally to the chromosomal loci where the desired mutation should be, as well as primers designed specifically to the antibiotic cassette. This procedure is described in great detail in chapter 3.

2.14.3 Eliminating the Antibiotic Resistance Gene

Elimination of the antibiotic resistance cassette from the chromosome after insertion by Red mutagenesis was completed following the method described by (Ellermeier, Janakiraman, and Slauch, 2002). Briefly, elimination of the antibiotic resistance cassette was performed using a temperature sensitive helper plasmid, pCP20 (Datsenko and Wanner, 2000), which encodes for the FLP recombinase (Cherepanov and Wackernagel, 1995). Strains containing a FRT Km FRT mutation were transformed with the helper plasmid, pCP20, recovered and plated onto LA plates at 30°C. Colonies

were restreaked at 30°C on LA-Amp plates. Individual colonies were then tested for loss of Km cassette by patching onto LA-Km plates.

Chapter 3 - Optimisation of λ Red Mutagenesis

3.1 Introduction to lambda Red Recombinase

Gene replacement in bacteria such as *S.* Typhimurium can, and has been achieved by a variety of methods. One of the easiest procedures for gene manipulation is to disrupt or replace the chromosomal DNA loci of interest with a gene encoding antibiotic resistance or other marker cassette. A general method that utilises this theory is the integration of a plasmid containing selectable markers and a mutated copy of the gene of interest by homologous recombination. The plasmid itself is normally resolved using culture conditions that it is unable to replicate in, for example the absence of the *pir* product (Donnenberg and Kaper, 1991) leaving the mutated copy of the gene. This method had been very successful, and the method of choice over a number of years. However there are a number of drawbacks associated with this procedure including lack of efficiency of integration and time taken, due to cloning and sub-cloning of the gene to produce a mutated copy in the vector of choice. Typically this method can take up to 6 months in *S.* Typhimurium, depending on the manipulability of the gene of interest.

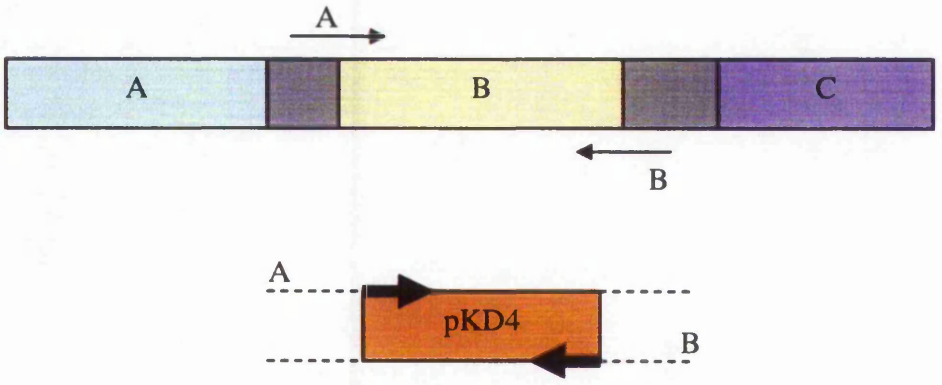
For many years genes in yeast have been directly disrupted using a PCR generated DNA fragment consisting of a selectable antibiotic resistance cassette flanked by a short region of DNA homologous to the gene of interest (Baudin *et al.*, 1993;Wilson, Davis, and Mitchell, 1999) . In contrast to yeast the majority of bacteria are not naturally competent for linear DNA, due to the presence of intracellular exonucleases encoded for by host *recBCD* which rapidly degrade the linear DNA before it can recombine (Lorenz and Wackernagel, 1994). In *recBCD*$^-$ strains evidence of transformation of linear DNA has been described (Cosloy and Oishi, 1973a;Cosloy and Oishi, 1973b), but these strains grow extremely poorly and are deficient in recombination. Bacteriophages encode their own extremely efficient homologous recombination system, known as red, which works by suppressing the bacterial RecBCD endonuclease (Smith, 1988). Using a

combination of this knowledge, (Datsenko and Wanner, 2000) developed a one step inactivation system of chromosomal genes in *E. coli* K12 using PCR products. To do this they constructed a temperature sensitive helper plasmid containing λ phage genes *exo*, *bet* and *gam* under control of an arabinose inducible promoter. Gam inhibits the RecBCD nuclease from attacking the linear DNA, and Exo and Bet generate recombination activity for that linear DNA. Their method used between 10-100ng of PCR generated linear DNA, which consisted of an antibiotic resistant cassette flanked on either side by 36 nucleotides of homology to the gene of interest. This was then electroporated into competent cells of *E. coli* expressing the phage Red genes, and the desired chromosomal disruption selected for by plating out onto the appropriate antibiotic. Using this method they reported the successful mutation of 13 different genes (Datsenko and Wanner, 2000). A schematic of this method is depicted in figure 1B.

3.2 Lambda Red Mutagenesis in *S.* Typhimurium Using the Exact Datsenko and Wanner Method

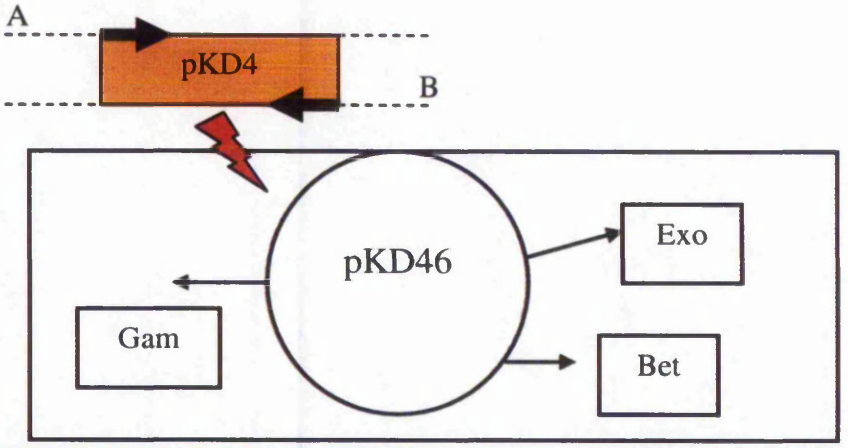
To enable the rapid construction of mutations in σ^E regulated genes we identified, the initial aim of this thesis was to use the Red method described above for mutant construction in *S.* Typhimurium. To start with the exact method described in (Datsenko and Wanner, 2000) was followed other than the oligonucleotides used were from MWG, using a chloramphenicol template plasmid (pKD3) with 36 nucleotides homology to *hisG* as a control gene. No colonies were achieved from the first 5 attempts with this method. Linear DNA with homology to *sbmA* and *bacA* (σ^E regulated genes) was also used in case there was a problem mutating *hisG* by this method. There was no success with the next 5 attempts using this linear DNA.

1.



Linear DNA is amplified using primers homologous to a template plasmid (pKD4) and with 40bp homology to the 5' and 3' ends of the gene of interest.

2.



Linear DNA is electroporated into bacterial cells containing a helper plasmid (pKD46) which have been expressing Bet, Exo and Gam under the control of arabinose.

3.

Post recovery kanamycin resistance colonies should have a kanamycin resistance cassette inserted in place of the gene of interest. This can be checked by using primers external to the loci of interest and also homologous to the resistance cassette to check orientation.

Figure 1B - Schematic diagram of the principle behind λ Red mutagenesis.

3.3 Arabinose Induction

From previous experience of inducing the pBAD promoter in wild type *S.* Typhimurium, strain SL1344 optimal expression from this promoter requires a higher concentration of L-arabinose in comparison with *E. coli* due to metabolism of arabinose. Figure 1 depicts growth of SL1344 harbouring helper plasmid pKD46 in various concentrations of L-arabinose. This was done to investigate the greatest concentration of arabinose we could use without causing too great an effect on growth kinetics. 1mM L-arabinose is the highest concentration that can used in this procedure without causing a toxic effect on the bacterial cells.

3.4 The First Success

Using the Datsenko and Wanner method and persisting with the *hisG* control gene, a 50ml LB culture was induced at time 0 with 1mM L-arabinose and grown to an OD600nm before being harvested for competent cells. 100ng of linear DNA consisting of 36 nucleotides of homology to the middle of *hisG* at the 5' and 3' ends, producing an insertion mutant, and a chloramphenicol cassette from pKD3 was electroporated into these cells. After recovery for 1 hour in 1ml SOC broth, 100µl were plated out onto LA-chloramphenicol plates. No colonies were present after 24 hours, so the remaining 900µl were plated out. After a further 24 hours 2 chloramphenicol colonies were present. Figure 2 shows the PCR verification of both of these colonies as *hisG*::Cm mutants with a chloramphenicol resistance gene.

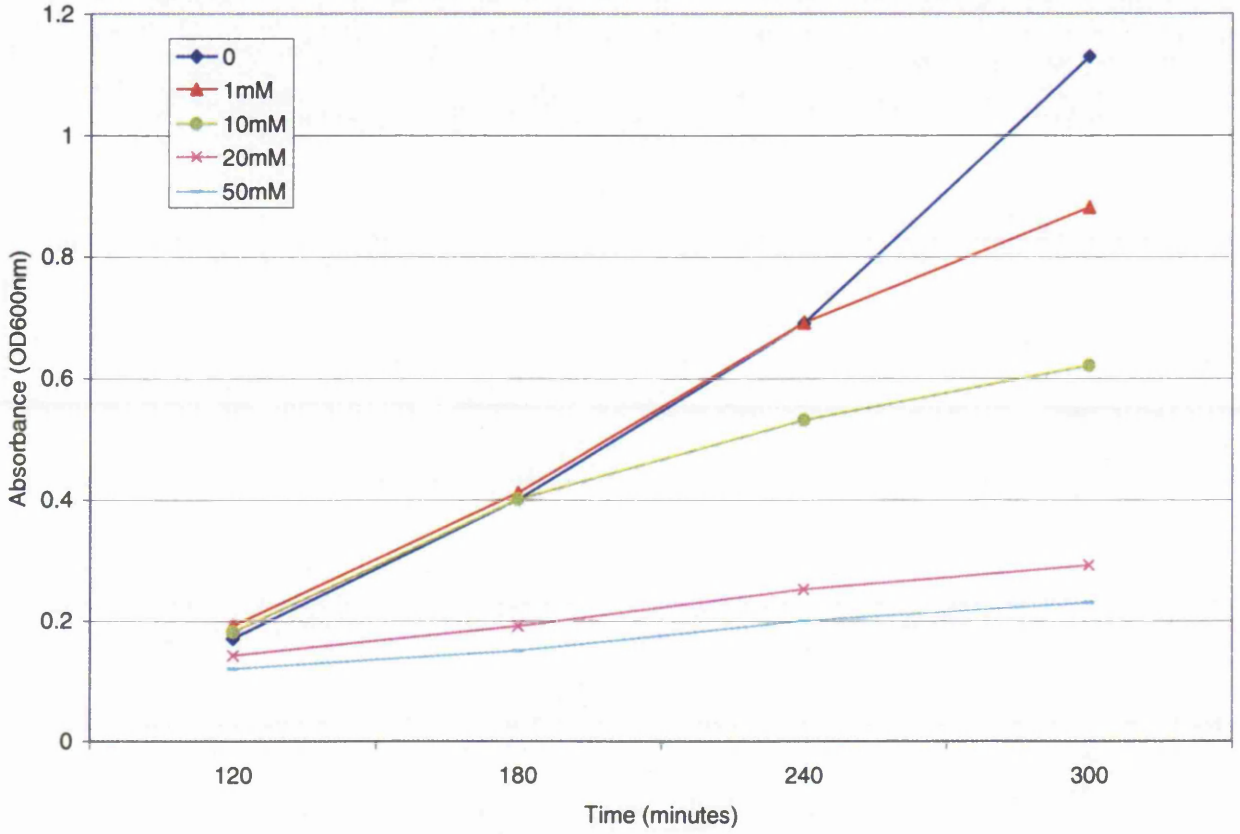


Figure 1. Effects of L-arabinose concentration in L-broth on growth of SL1344 carrying plasmid pKD46.
Data presented here is a mean of three independent 50ml cultures. The appropriate concentration of L-arabinose was added at time 0.

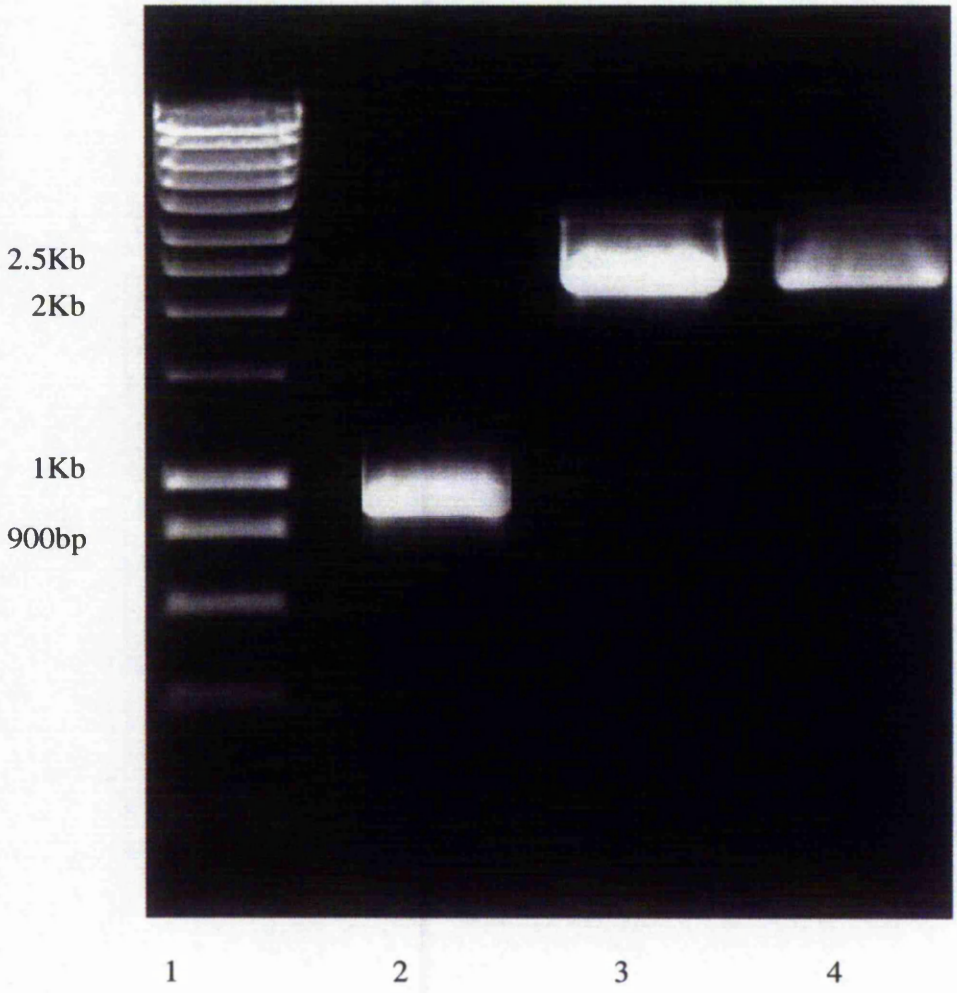


Figure 2. PCR verification of putative SL1344 *hisG*::Cm mutants.

Boilate PCR were performed on WT SL1344 **(2)**, HisG1 **(3)** and HisG2 **(4)** using oligonucleotides HisGEXtF and HisGExtR. The band from the WT strain of 900bp is in agreement with that expected. HisG1 and HisG2 bands are ~2400bp corresponding to the insertion of the 1.5Kb chloramphenicol cassette in *hisG*. Sizes are approximated from Hyperladder I DNA ladder (Bioline) **(1)**.

Unfortunately this success could not be repeated either with the same PCR product used in this mutagenesis or with *sbmA* and *bacA*, using linear DNA containing either a chloramphenicol or kanamycin cassette. The big problem at this stage was the high number ($\geq$ 50) of false Km^R positive colonies that were consistently isolated on L-agar Km plates (containing 50μg/ml Kanamycin; Sigma) when using Km linear DNA. This was very confusing as samples were treated with *dpnI* to remove the original template plasmid, which seemed to be successful on agarose gel analysis. Also this phenomenon was not observed with the chloramphenicol containing PCR products.

3.5 Identification of Important Factors

After discussion with Dr. Junkal Garmendia and Dr. Derek Pickard at Imperial College, London we identified a number of differences between the method they had been successfully using and the method we had been following based on the *E. coli* publications.

Firstly the type of L-broth used to culture the *S.* Typhimurium strain harbouring the helper plasmid differed from ours. We were using the standard Luria Bertani preparation (Sambrook, Fritsch, and Maniatis, 1989) using components supplied by Oxoid, whilst they were using a ready mix Lennox broth preparation from Invitrogen.

Secondly the amount of DNA used was far greater than the amounts recommended in *E. coli*. In *E. coli* between 1 and 100 ng of linear DNA product was recommended, whilst at Imperial although not quantified they were using up to half of 1200μl PCR reaction concentrated into 30μl. This contains DNA in the μg rather than ng region. The reaction composition and cycle conditions for generation of the linear DNA product was very similar to ours as well as the digest and clean up procedure used.

Thirdly, the source of oligonucleotides differed to ours, although the length of homology to the target locus concurred. We had been routinely using oligonucleotides synthesised by MWG for all aspects of our molecular work, whilst they had been using oligonucleotides synthesised by Sigma Genosys.

Fourthly, the source and concentration of kanamycin differed. They were using a concentration of 75μg/ml supplied by Invitrogen as the quality of kanamycin from Sigma was of some concern to these groups.

Finally, the background strain of *S.* Typhimurium carrying the Red helper plasmid was 12023 rather than the SL1344 strain we had been using.

3.6 Optimisation of the Glasgow Method

After incorporating the differences documented above into our previous protocol we attempted Red mutagenesis of *sbmA* and *bacA* in the 12023 *S.* Typhimurium background, completely replacing the CDS with a kanamycin cassette. Immediate success was achieved for both these genes. Figure 3 depicts the *S.* Typhimurium strain 12023 *sbmA*::kan mutant. A number of different changes had been made to the protocol, in comparison with our original method based on that described for *E. coli*. It was therefore important to try and identify the rate limiting factors in this procedure in case of any future problems.

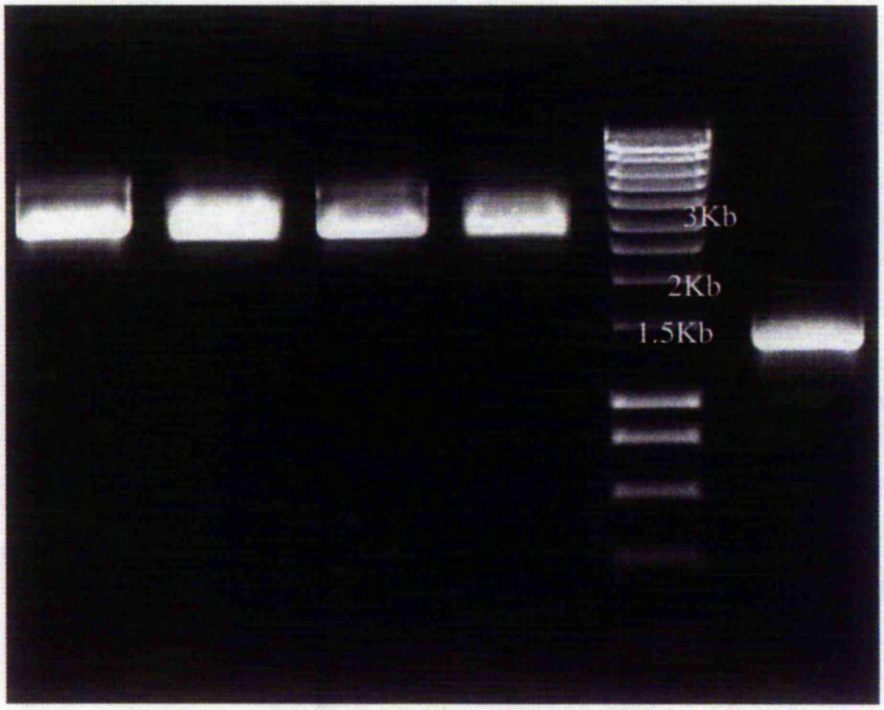


Figure 3. PCR verification of a *S.* Typhimurium strain 12023 *sbmA*::Kan mutant.

PCR verification was performed as in figure 2, using oligonucleotides SbmAExtF and SbmAExtR primers. The mutant size ~3Kb **(A1-A4)** corresponds with a 1.5Kb kanamycin cassette inserted into the 1.45Kb wild type copy **(WT)** of *sbmA* as determined by Hyperladder I DNA ladder **(L)**.

3.6.1 DNA Concentration

Figure 4 shows the number of positive Δ*sbmA* mutants isolated in relation to the amount of DNA electroporated into the competent cells of 12023 harbouring pKD46. 10µl of the DNA used here is equivalent to ~1µg. As you can see there is a definite optimum DNA concentration. The DNA concentration and positive colonies recovered appears to follow a normal distribution, where too little DNA is insufficient for recombination to occur but too much DNA either becomes toxic to the cells or overloads the recombination system preventing success. However we recommend that a variety of DNA concentrations between 100ng and 1.5µg are always used for each mutagenesis experiment.

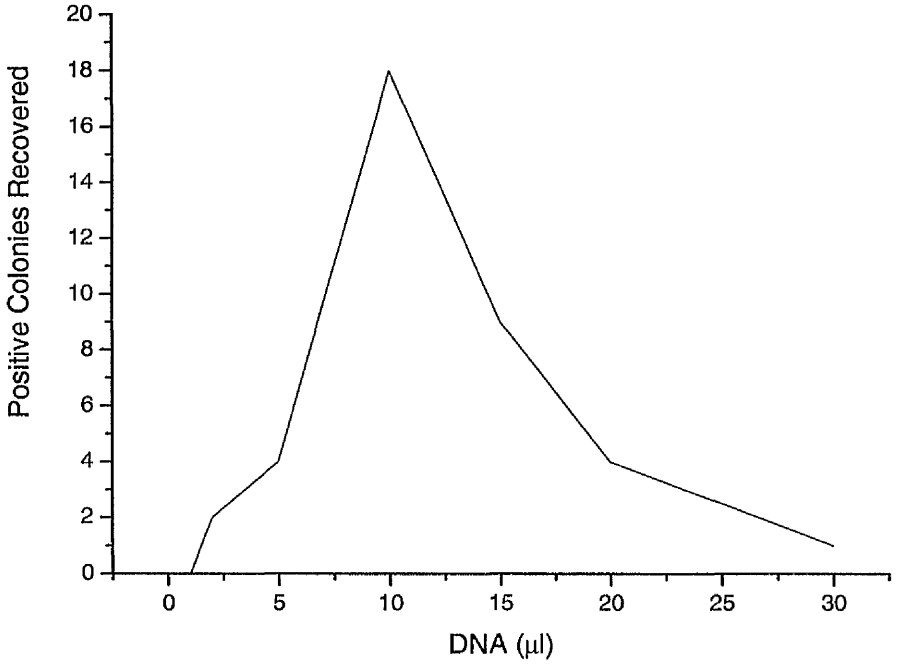


Figure 4. Graph showing correlation between quantity of DNA and positive colonies isolated.
Lambda red mutagenesis was performed using the amount of DNA (µl) as depicted on the x axis after which the number of colonies containing the desired mutation was confirmed by PCR, y axis.

3.6.2 L-Broth Composition

To assess the importance of the difference in composition of the liquid media used to culture the competent cells, 12023 was cultured in both Lennox and Miller L-broth. 10µl of the same batch of linear DNA targeting *sbmA* was used for mutagenesis. Surprisingly, 10 fold more positive colonies were recovered when bacteria were cultured in Lennox broth. The only difference between the two broths is Lennox broth uses peptone rather than tryptone and contains 5g litre less NaCl. Growth of wild type *S.* Typhimurium, strain SL1344 at 37°C in these two broths appears identical.

3.6.3 Background Strain

Identical protocols were followed using Lennox broth and 10µl linear DNA targeting *sbmA,* for both strains SL1344 and 12023 harbouring pKD46. Ideally we wanted to make the disruptions directly in SL1344 as this is the background strain we have historically used within the laboratory. Only 1 colony of Km resistant SL1344 was recovered compared to 25 colonies for 12023. Although the SL1344 colony contained the correct mutation, this huge difference in recombination frequency between the two strains was quite remarkable. For all future lambda Red mutations we used the 12023 strain to make the mutation which was subsequently inserted into SL1344 using P22 transduction.

3.6.4 Final Protocol

1. Design oligonucleotides with 40bp homology to the gene of interest on the forward and reverse primer.

2. PCR using the desired template vector pKD3 (Cm) or pKD4 (Kan). Need to use ~1μg DNA.

3. Pool the PCR reactions and clean up using 2 QiaQuick PCR clean (Qiagen) tubes (600μl reaction through each tube).

4. Elute the DNA with MQ water, 75μl per column, and pool (150μl total).

5. Add 15μl *dpn*I reaction buffer and 2μl *dpn*I. Incubate at 37°C for 3hrs.

6. Clean up the digest reaction with QiaQuick tubes.

7. Grow a 5ml overnight culture of 12023 carrying pKD46 in LB with 100μg/ml ampicillin at 30°C.

8. Dilute the O/N culture 1/100 into Lennox broth containing 100μg/ml ampicillin and 1mM L-arabinose.

9. Grow the cells to an OD600nm of 0.6, and pellet at 4°C.

10. Wash the cells 2x in ice cold sterile distilled water and 1x in sterile 10% glycerol. Resuspend the final pellet in 1ml of 10% glycerol.

11. Electroporate 0, 2, 5, 10(1μg), 20, 30 μl of DNA into 100μl cells, using 2mm cuvettes. Electroporation conditions used are the standard ones for the background strain (i.e *S.* Typhimurium – 600Ohms, 25μF, and 1.75Kv).

12. Recover the cells in 1ml of SOC at 37^0C for two hours.

13. Plate out 100μl of cells onto LB-agar containing the desired antibiotic.. Leave the remaining 900μl on the bench O/N.

14. If no colonies are isolated spin down the remaining 900μl and resuspend in 100μl SOC. Plate out the whole 100μl.

15. Check antibiotic resistance colonies have the desired mutation, using external PCR primers.

Chapter 4 - Regulation of the Extracytoplasmic Stress Response in *S.* Typhimurium

4.1 Introduction

The difference in lifestyles between *E. coli* and *Salmonella*, and indeed the differing environments they encounter throughout these lifestyles may suggest a requirement for differential regulation of stress responses between these closely related species (Winfield and Groisman, 2003). However the majority of knowledge regarding the regulation of Gram negative responses is derived from characterisation of a non-pathogenic laboratory strain of *E. coli*, whose response to stress is extrapolated solely from data achieved under laboratory conditions. It is difficult to accept that the regulon members and regulation of the ESR of *E. coli* and *S.* Typhimurium are identical, especially since we have already seen that the requirement for one arm of the ESR, the σ^E regulon, for cell viability differs (Humphreys *et al.*, 1999). We were keen to ascertain any differences in regulation of the *S.* Typhimurium *rpoE* regulon and any interactions that this regulon has with other regulatory networks.

The σ^E regulon and the *cpxAR* two component regulator are known to respond to distinct periplasmic signals which initiate an upregulation in their transcription but still overlap in their regulation of the serine protease, *htrA* (Connolly *et al.*, 1997;Raivio and Silhavy, 1999). More recently (De Wulf *et al.*, 2002), have reported that in *E. coli* the absence of *cpxR* results in slightly increased levels of *rpoE* mRNA, where as a constitutively active *cpx* mutant (*cpxA**) results in very low levels of *rpoE* mRNA, showing that at least in *E. coli* there is a degree of cross regulation of the ESR pathways at the transcriptional level. Another interaction at the transcriptional level has been described between Cpx and another stress response regulatory network. In *E. coli* an increase in transcriptional activation of *cpxRA* is observed during onset of stationary

phase. This is due to transcription of *cpxRA* by the stationary phase alternative sigma factor, RpoS (De Wulf, Kwon, and Lin, 1999;Raivio, Popkin, and Silhavy, 1999).

Although this study predominantly focuses on characterisation of the *S.* Typhimurium σ^E regulon, to fully characterise the interactions between *rpoE* and *cpx*, we have also investigated the regulation of the *S.* Typhimurium *cpx* mediated envelope response to try and fully complete the regulatory picture.

4.2 Construction of *cpx*, *rpoE* and *rpoH* Promoter Reporter Plasmids.

The regions to be fused with *lacZ* were PCR amplified using the appropriate oligonucleotides and directionally cloned into pTL61t on *Hind*III and *Xba*I restriction enzymes sites incorporated into the oligonucleotides (figure 5). pTL61t contains a promoterless copy of *lacZ*. Confirmation of cloning involved three methods; restriction digestion, PCR with oligonucleotides homologous to the regions flanking the cloning site of pTL61t and nucleotide sequencing. The regulatory regions of *cpx*, *rpoE* and *rpoH* which are cloned into pTL61t are depicted in figure 6.

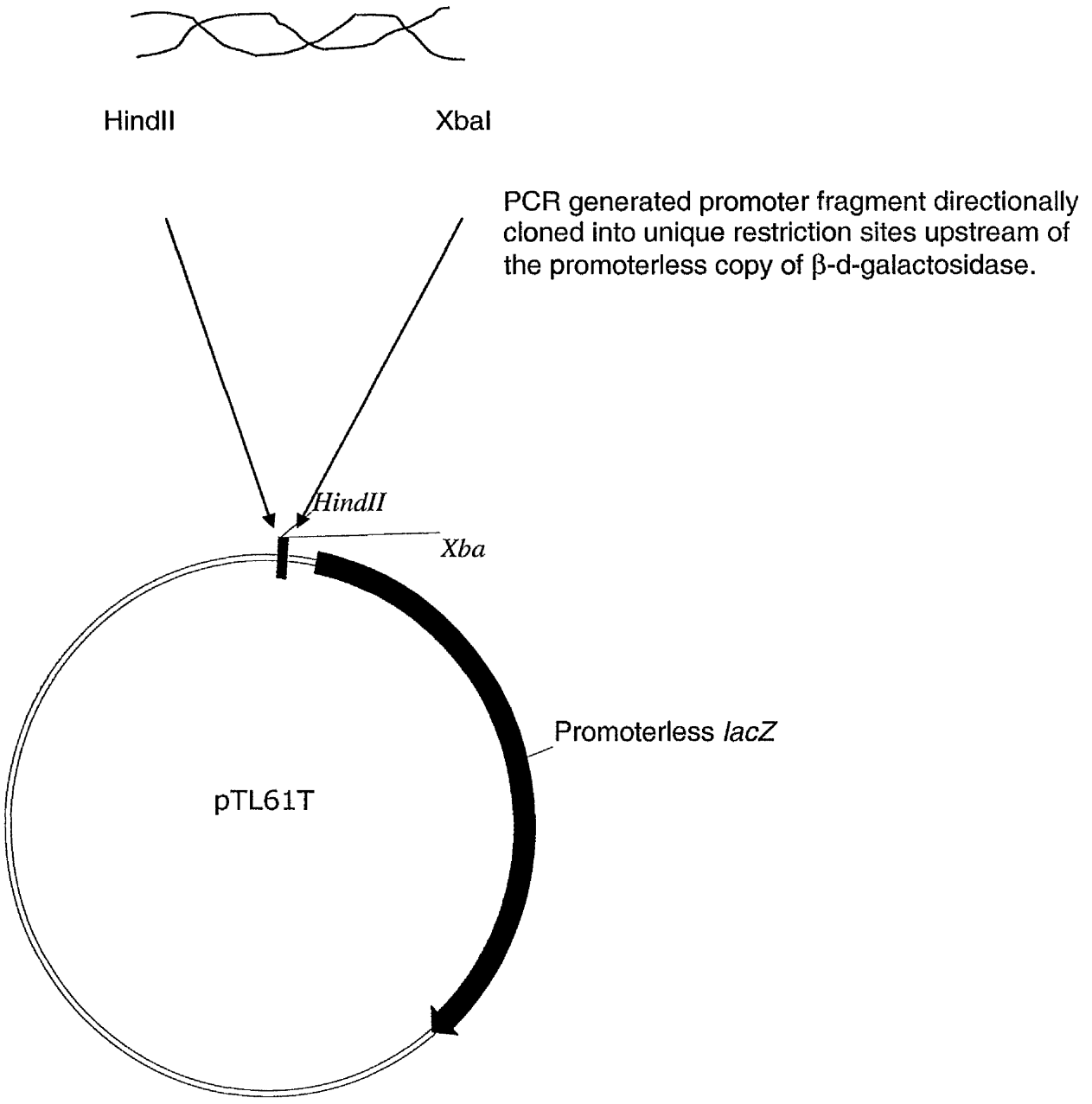
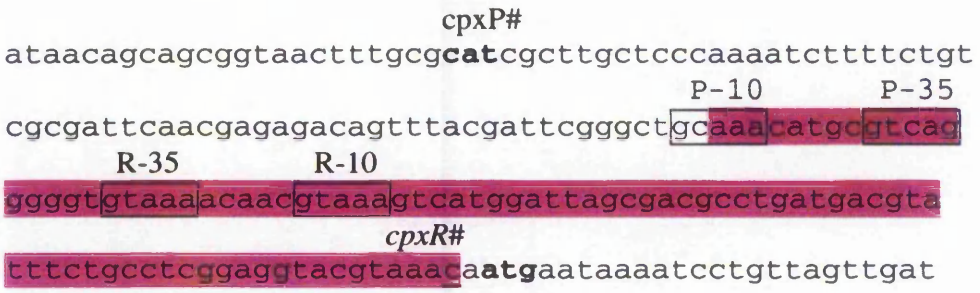


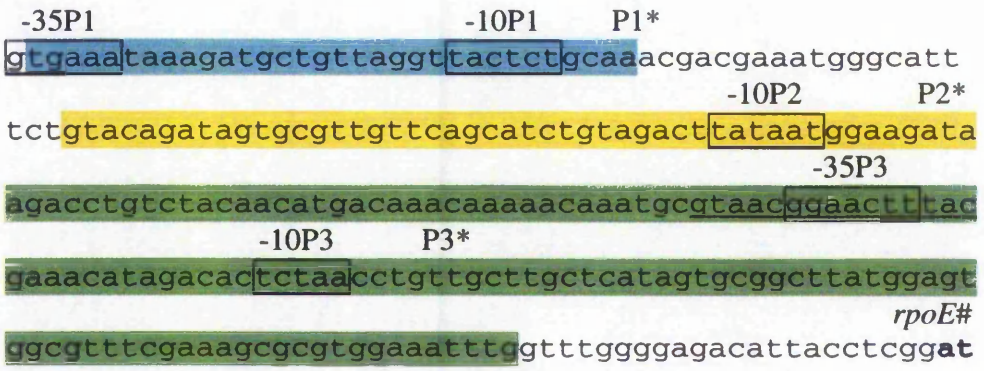
Figure 5. Construction of promoter-*lacZ* transcription fusions

PCR generated fragments containing 5' *HindIII* and 3' *XbaI* restriction sites were directionally cloned upstream of a promoterless copy of *lacZ*. Confirmation of cloning was based on blue-white selection, PCR using primers pTL61tF and R, restriction digest analysis and nucleotide sequencing.

A) Regulatory Region Between *cpxP* and *cpxR* (pcpxRs)



B) Regulatory Region of *rpoE* (prpoEP1, prpoEP2 and prpoEP3)



C) Regulatory Region of *rpoH* (prpoHpall)

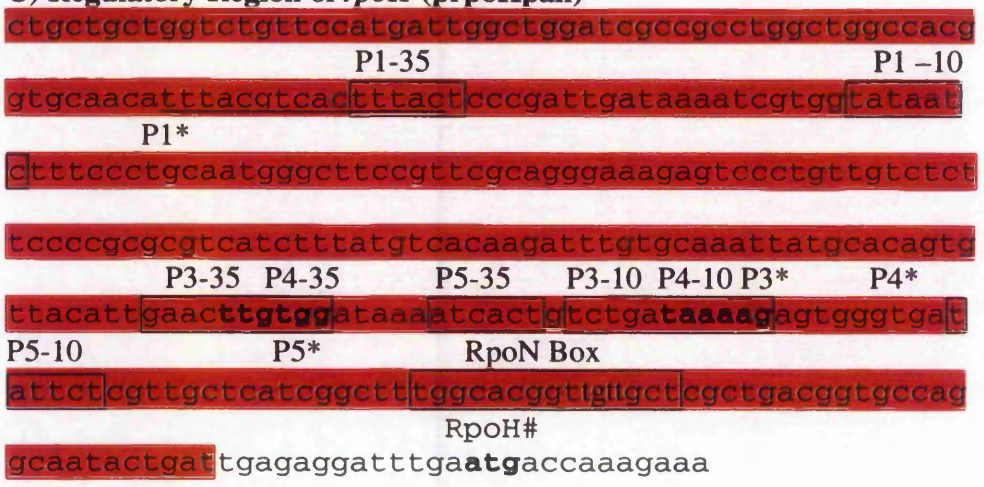


Figure 6. Regulatory regions of 3 stress response regulators (individual cloned regions highlighted).

The regulatory regions of A) *cpx*, B) *rpoE* and C) *rpoH* are depicted above. Promoter and binding sites, as well as transcriptional start points (tsp) for *cpx* and *rpoH* are predicted from *E. coli* (Nagai *et al.*, 1990;Kallipolitis and Valentin-Hansen, 1998;Pallen, 1999;Ramirez-Santos *et al.*, 2001;Solis-Guzman *et al.*, 2001;De Wulf *et al.*, 2002), and confirmed in *S.* Typhimurium for *rpoE* (Miticka *et al.*, 2003). Non uniform underscore represents a CpxR-P box. Bold text in boxed regions indicates an overlap of two sites. * indicates tsp, # indicates start codon.

4.3 Transcription of *rpoE* is Governed by Three Promoters

In *E. coli* the *rpoE* operon is regulated by two promoters, a more upstream promoter designated P1 and a P2 promoter autoregulated by σ^E (Raina, Missiakas, and Georgopoulos, 1995;Rouviere *et al.*, 1995). The P2 promoter was identified in an identical position in both papers whilst a differing consensus for the beginning of the P1 promoter was drawn. To identify the promoters of *rpoE* in *S.* Typhimurium, the Kormanec laboratory using S1-nuclease mapping identified 3 promoters upstream of *rpoE* (Miticka *et al.*, 2003). Transcription from the *rpoE*P1 promoter was highest during exponential phase, but decreased during stationary phase. Transcription from the *rpoE*P2 promoter was fairly consistent throughout the growth phases, although mRNA levels were low. The *rpoE*P3 promoter was fairly inactive during exponential phase but increased dramatically at the end of exponential phase and mRNA levels remained high throughout stationary phase. The positions of the *S.* Typhimurium *rpoE*P1 and *rpoE*P3 correspond to the *E. coli* P1 and P2 promoters (figure 6). Although not identified as a promoter in *E. coli* the sequence for the *S.* Typhimurium *rpoE*P2 promoter is present in *E. coli*, and a weak signal corresponding to this sequence has been observed (Rouviere *et al.*, 1995). The RpoE operon of *S.* Typhimurium and the location of the promoters is depicted in figure 6A. Using these *S.* Typhimurium *rpoE* promoter sequences provided to us by the Kormanec laboratory, we constructed transcriptional fusions to *lacZ*, to confirm their findings and to analyse the activity of these promoters in wild type and mutant *S.* Typhimurium strains in different environments. We also used the autoregulated *rpoE*P3 construct as a tool to analyse activation of the *S.* Typhimurium σ^E pathway and to observe interactions with other regulatory pathways by measuring activity in a wide range of genetic backgrounds.

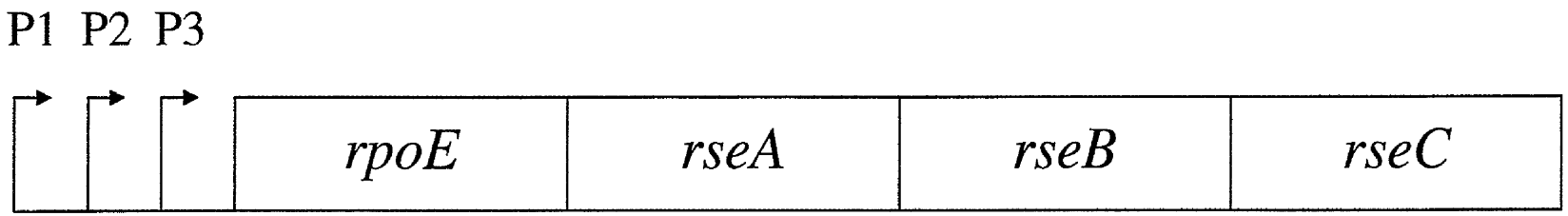
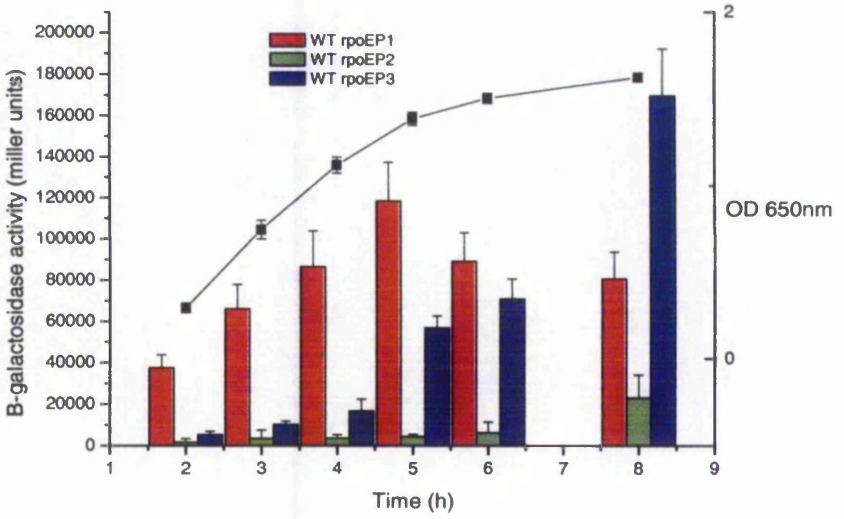


Figure 6A – Operon organisation of the RpoE regulon of *S.* Typhimurium.

Three promoters have been implicated in the regulation of the RpoE operon in *S.* Typhimurium, unlike in *E. coli* where only two promoters were identified. . The arrangement of the genes within the *S.* Typhimurium operon is identical to that identified in the majority of enteric bacteria.

A)



B)

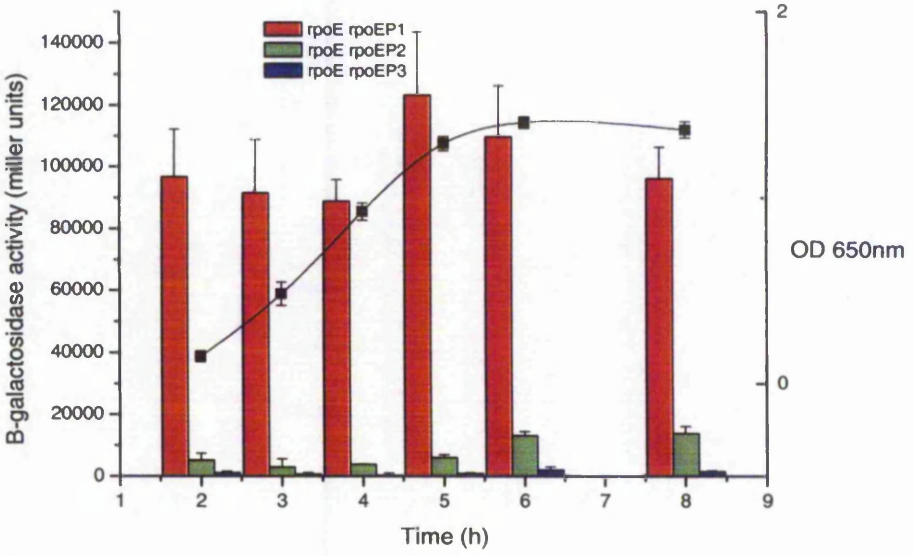
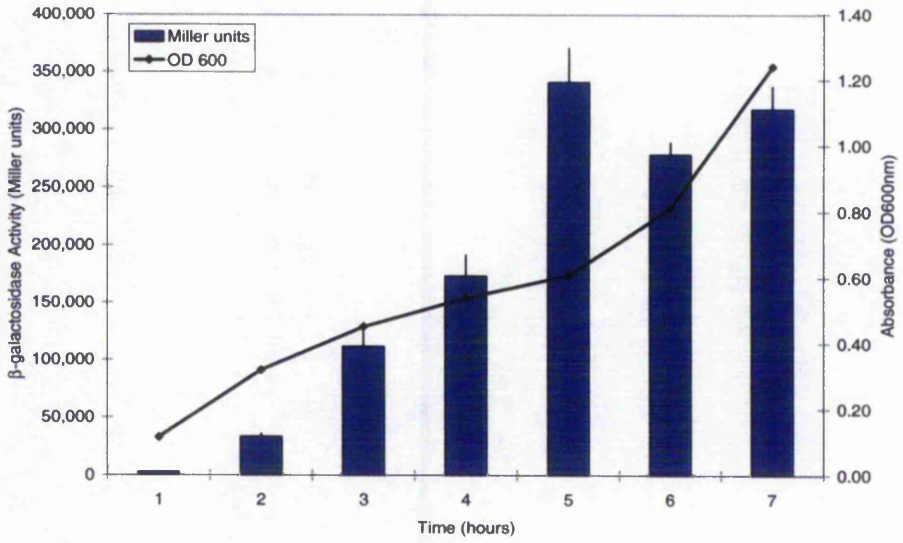


Figure 7. Comparison of activity of the three *rpoE* promoters in both wild-type S. Typhimurium SL1344 (A) and an *rpoE* mutant GVB311 (B). Cultures were grown overnight and then diluted 1/100 into fresh LB medium. β-galactosidase assays were performed at the time points shown, and the activity determined using the arbitrary but well recognized Miller units (Miller, 1972). Each point represents the mean of seven assays, and error bars indicate the standard deviation from the mean.

Figure 7 depicts the results from the β-galactosidase assays performed on both wild type *S.* Typhimurium SL1344 and an *rpoE* mutant (GVB311) harbouring the three different *rpoE* promoters. The *in vivo* data shown here correlates well with that observed *in vitro.* The *rpoE*P1 promoter is the most active promoter during lag phase and exponential phase. Interestingly in the absence of σ^E, the *rpoE*P1 promoter exhibits higher levels of activity than in the wild type SL1344 strain throughout the growth phases. The *rpoE*P2 promoter exhibits low levels of activity throughout the growth phases and the presence or absence of σ^E seems to have no affect on its activity. The *rpoE*P3 promoter is completely inactive in the *rpoE* mutant and is relatively inactive up to late exponential phase in the wild type strain; however upon entry into stationary phase this σ^E dependent promoter becomes hyperactive, consistent with stationary phase being an inducing condition of the σ^E regulon (Nitta *et al.*, 2000).

Further confirmation of the auto regulation of the *rpoE*P3 promoter by σ^E itself is shown in figure 8. Using plasmid pAC-*rpoE*ST4 containing the *S.* Typhimurium *rpoE* gene under the control of p_{BAD} promoter, in an *rpoE* mutant background we can control the presence or absence of σ^E within the bacterium using induction with arabinose or repression with glucose. In this strain carrying the *rpoE*P3 *lacZ* fusion, we can investigate the effects of σ^E overexpression on the transcriptional activity of the *rpoE*P3 promoter. Figure 8 clearly shows that in the presence of arabinose where *rpoE* is being overexpressed from the plasmid, the *rpoE*P3 promoter becomes hyperactive after around 5 hours growth, although greater levels of transcription are seen at all time points in the presence of arabinose

A)



B)

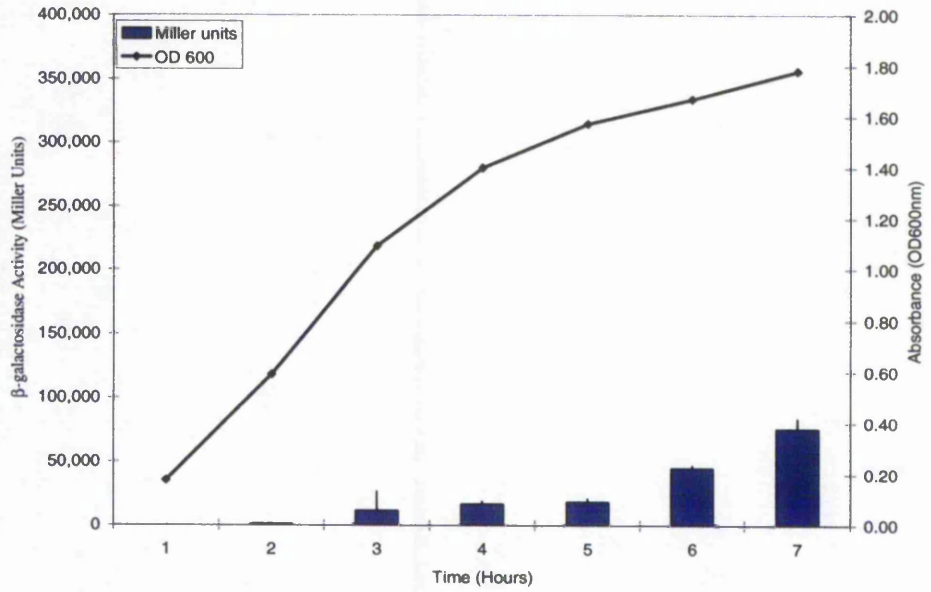


Figure 8. Effects of over-expressing σ^E on the *rpoEP3* promoter.

The β-galactosidase assays were performed as in figure 4 but with an *rpoE* mutant strain harbouring both plasmids p*rpoE*P3 and pAC-*rpoE*ST4 in the presence of A) 0.2% L-arabinose and B) 0.2% glucose.

compared with glucose. There is an increase in transcriptional activity seen in the strain grown in the presence of glucose, after approximately 3 hours, which suggests some leakage from the plasmid after glucose depletion due to metabolism.

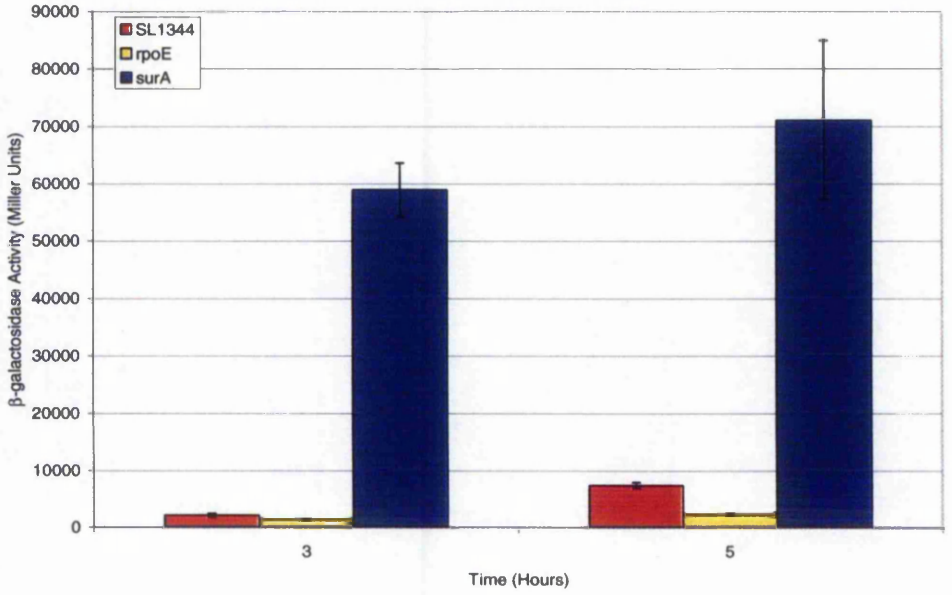
4.4 Regulation of the Auto-regulated *rpoE* Promoter, *rpoEP3*.

In figures 7 and 8 we have already shown that the *rpoE*P3 promoter in *S.* Typhimurium is auto-regulated by σ^E itself, and is induced under the known activating condition of entry into, and survival within stationary phase. In *E. coli* the autoregulated P2 promoter of *rpoE* and the σ^E regulated P3 promoter of *rpoH*, have been used as tools to measure regulation and activity of the σ^E regulon *in vitro*. We have adopted this same approach using the *S.* Typhimurium *rpoE*P3 promoter to ascertain any further differences between the regulons of these closely related bacteria. In *E. coli*, induction of the σ^E regulon is thought to result from the accumulation of misfolded or denatured outer membrane proteins within the bacterial periplasm. This phenotype can also be mimicked by genetic manipulation of the bacteria which also results in the build up of misfolded proteins within the periplasm, usually through loss of periplasmic proteases, peptidyl prolyl isomerases or proteins with chaperone function. The absence of SurA (Missiakas, Betton, and Raina, 1996;Rouviere and Gross, 1996), Skp (Missiakas and Raina, 1998), FkpA (Missiakas, Betton, and Raina, 1996) and PPiD (Rouviere and Gross, 1996) are all known to induce the *rpoE* regulon of *E. coli* as does the absence of DsbA in *E. coli* and *S.* Typhimurium (Testerman *et al.*, 2002). This may indicate that alteration of periplasmic redox conditions also induces σ^E but may still be as a result of misfolded proteins. We were keen to measure the effect of similar mutations on the activity of the *S.* Typhimurium σ^E pathway.

Figure 9 depicts the data obtained from β-galactosidase assays performed on *surA* and *skp* minus strains harbouring the *rpoE*P3 reporter construct. The absence of SurA is by far the greatest inducer of the σ^E regulon of these two strains, with a 12 fold induction seen during mid-exponential phase in comparison with the wild type strain. This is a good background strain to use for screening of putatively σ^E regulated promoters, and appears to be specific as an increase in activity from the empty vector and from non-σ^E regulated genes is not seen in this strain (GR-data not shown). However, clear induction of the σ^E regulon is observed in a *skp* minus background, but at a level of 5 fold induction in comparison with the wild type strain. Interestingly this type of induction is not seen in all strains that have a function associated with maintaining protein homeostasis within the periplasmic space. Mutations resulting in loss of functional FkpA and HtrA do not induce the *rpoE* regulon in *S.* Typhimurium (GR, data not shown).

(Testerman *et al.*, 2002) have shown that σ^E plays a distinct but complementary role to σ^S in stationary phase survival of *S.* Typhimurium on the premise that strains lacking both of these alternative sigma factors survive less well than the single mutants. We investigated the activity of the *rpoE*P3 promoter in an *rpoS* mutant background. No difference in activity of the *rpoE*P3 promoter is observed between the wild type strain and an *rpoS* minus strain during exponential or stationary phase growth. Similar interactions between the *rpoE* regulon and stringent response have also been proposed (Ades, 2004), as a possible link between the σ^E and RpoS regulated pathways. During this review they refer to unpublished data which indicates that growth phase activation of σ^E in *E. coli* does not requires RseA, but depends on the alarmone ppGpp. In a *S.* Typhimurium *relA/spot* mutant background lacking functional ppGpp, no difference in

A)



B)

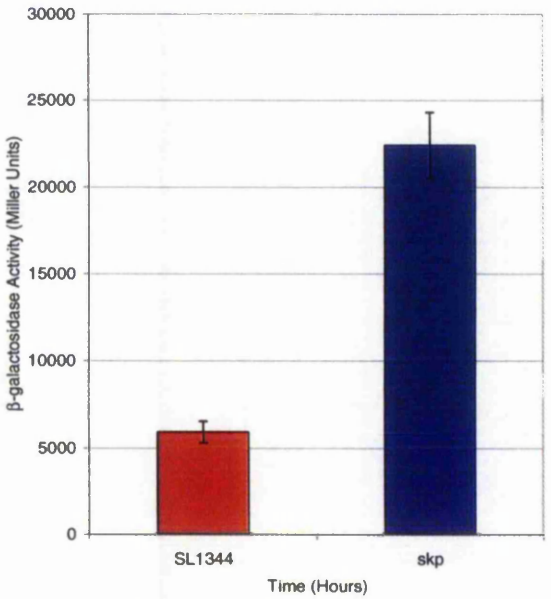


Figure 9. Effects of genetically engineered periplasmic disruption on *rpoE* induction.

β-galactosidase assays were performed on A) WT *S.* Typhimurium and *rpoE*, *surA* mutants harbouring plasmid p*rpoE*P3 at 3 and 5 hour time points of a growth curve and B) WT *S.* Typhimurium and a *skp* deletion mutant harbouring the same plasmid at 5 hours into a growth curve. 3 hours is equivalent to mid-exponential phase and 5 hours stationary phase.

activity of the *rpoE*P3 promoter is observed in comparison with the wild type strain throughout any phase of growth (GR, data not shown).

4.5 Activity of the *rpoEP3* Promoter within Murine Macrophages

The promoter region of the *S.* Typhimurium σ^E regulated gene *htrA* has been transcriptionally linked with *lacZ* and its activity assayed within a number of mammalian cells (Everest *et al.*, 1995). In all cell types tested, which included Hep-2, Caco-2 and THP-1 macrophages, expression of *lacZ* from the *htrA* promoter was significantly enhanced. With this in mind we compared the activity of the three *rpoE* promoters within RAW 264.7 murine macrophages (figure 10). As with all conditions analysed the activity of the *rpoE*P2 promoter was the lowest of the three, but did demonstrate a degree of activation after 24 hours post infection. The *rpoE*P3 promoter exhibited a 16 fold increase in activity between 4 and 24 hours post infection, with the *rpoE*P1 promoter following a similar trend with a 10 fold induction. The increase in activity of the *rpoE*P3 promoter observed was as expected due to the known requirement for functional σ^E in the intracellular survival of *S.* Typhimurium (Humphreys *et al.*, 1999). However, the increase in, and indeed the degree of activity seen with the *rpoE*P1 promoter was quite surprising. This could possibly be explained due to the *rpoE* dependent induction of *rpoD* (Dartigalongue, Missiakas, and Raina, 2001;Miticka *et al.*, 2003).

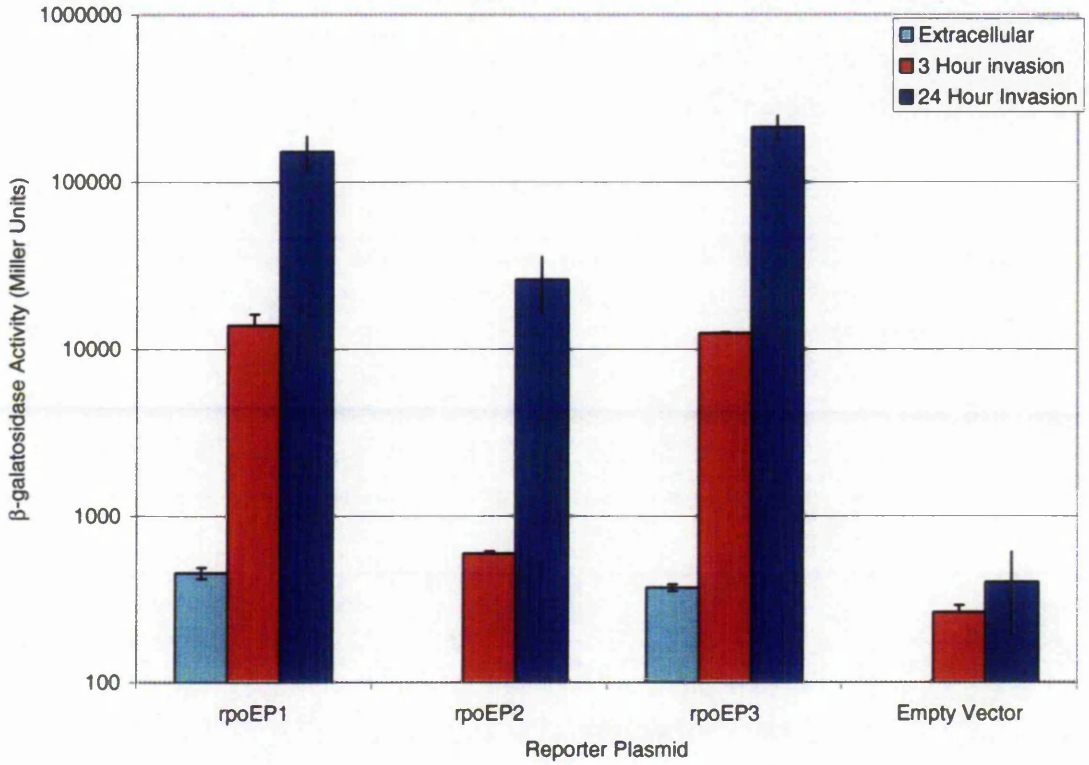


Figure 10. Transcriptional activity of the three *rpoE* promoters post infection of RAW264.7 murine macrophages. WT *S.* Typhimurium strains harbouring the three *rpoE* promoter fusions or the empty vector control (pTL61t) were used to infect macrophages with a MOI ~1. Activity of the promoters in the extracellular environment was measured prior to gentamicin killing. Activity of promoters in the invaded cells was established post cell lysis with 0.1% Triton and the lysate used in a β-galactosidase assay. Bacterial numbers were ascertained by viable count and extrapolated to a *S.* Typhimurium standard curve to achieve the equivalent OD600nmm value.

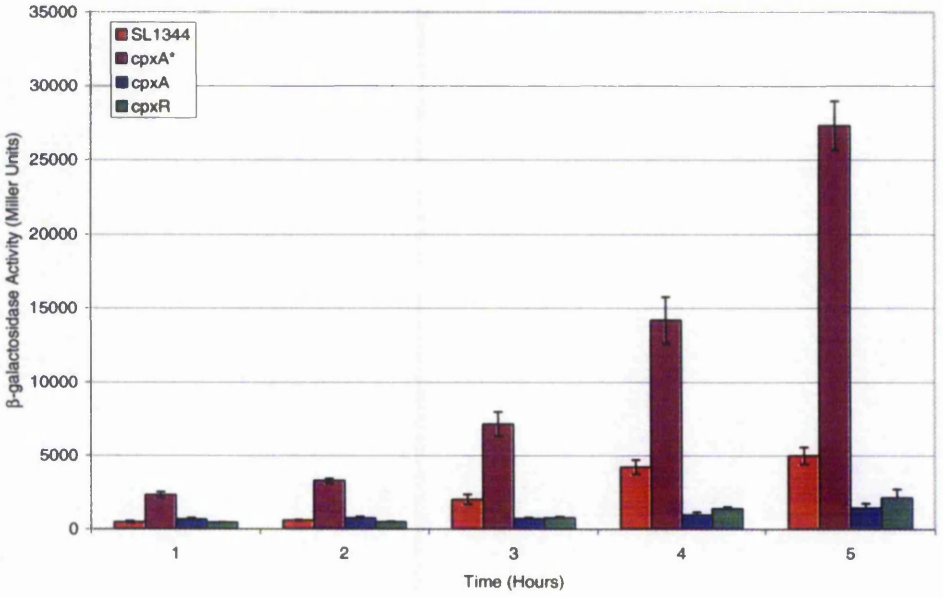
4.6 Activity of the Cpx Regulated *cpxRA* Promoter, p*cpxR::lacZ*

Overlap in regulon members of the *rpoE* and *cpx* pathways have been described in *E. coli* (De Wulf *et al.*, 2002) (Miticka *et al.*, 2003). To ascertain regulation of the Cpx regulated arm of the ESR in *S.* Typhimurium we constructed a *lacZ* reporter fusion to the Cpx-dependent *cpxRA* promoter (*pcpxR::lacZ*). This construct was validated as a tool for measuring induction of the *cpx* pathway via assaying the β-galactosidase activity of this construct in a variety of *cpx* mutant backgrounds, namely *cpxA* and *cpxR* null mutants and a constitutively active, signal blind *cpxA** mutant. Figure 11 shows the results of these assays, with activity of the construct in the null mutant backgrounds similar to that seen with empty vector. Throughout all phases of the growth curve the *cpxR* promoter in the *cpxA** background is significantly more active than in the wild type strain, although an increase in expression does occur as growth progresses.

The *cpxR* promoter construct was also used as a tool for validation of the *cpxA** mutant. The *cpxA** mutant exists as two colony phenotypes, 'big' and 'small', but it appears that the *cpxA** small phenotype is the correct signal blind mutant, whilst the big phenotype that occurs after one growth cycle is no longer a constitutively active *cpxA** strain. From the levels of promoter activity observed we hypothesise that the big colony phenotype contains a secondary mutation.

A two fold increase in activity of this promoter is also seen in the wild type strain between mid-exponential and stationary phase growth. Such an increase in *cpxRA* expression has been described in *E. coli* (Raivio, Popkin, and Silhavy, 1999) and has been directly attributed to regulation of *cpx* by RpoS. We measured activity

A)



B)

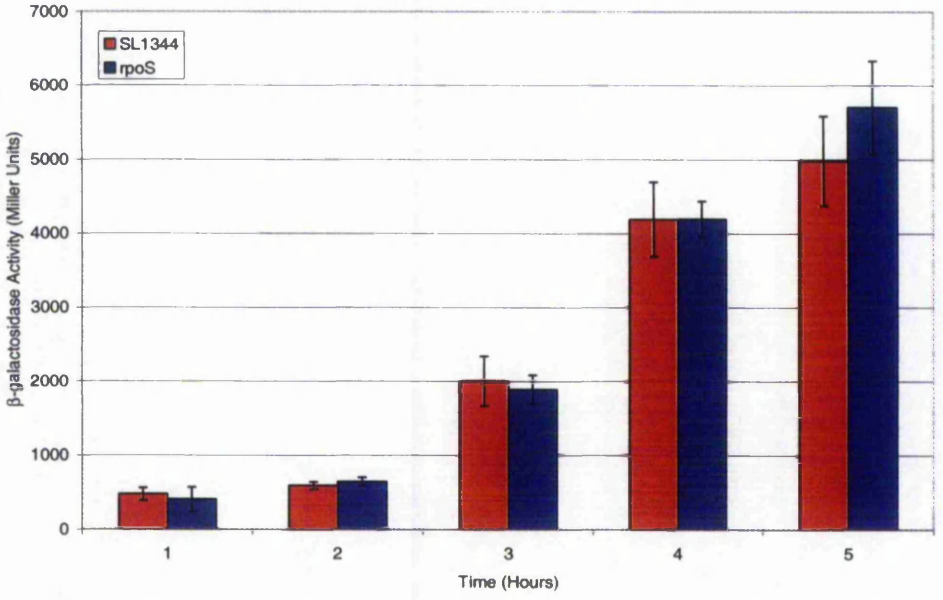


Figure 11. Effect of mutations in stress response regulators on expression of the *cpx* genes.

β-Galactosidase activities were determined for A) *cpx* mutants of serovar Typhimurium strains harbouring the *pcpxR::lacZ* reporter plasmid, and B) an equivalent *rpoS* mutant. Strains were grown for 5 h (until onset of stationary phase) in LB broth at 37°C with aeration. Bars represent means for seven replicates; error bars are standard deviation of the means.

of the *cpxR* promoter in an *S.* Typhimurium *rpoS* mutant background. Figure 11 illustrates that there is no difference in *cpxRA* expression between the wild type strain and an *rpoS* mutant background throughout the growth phases.

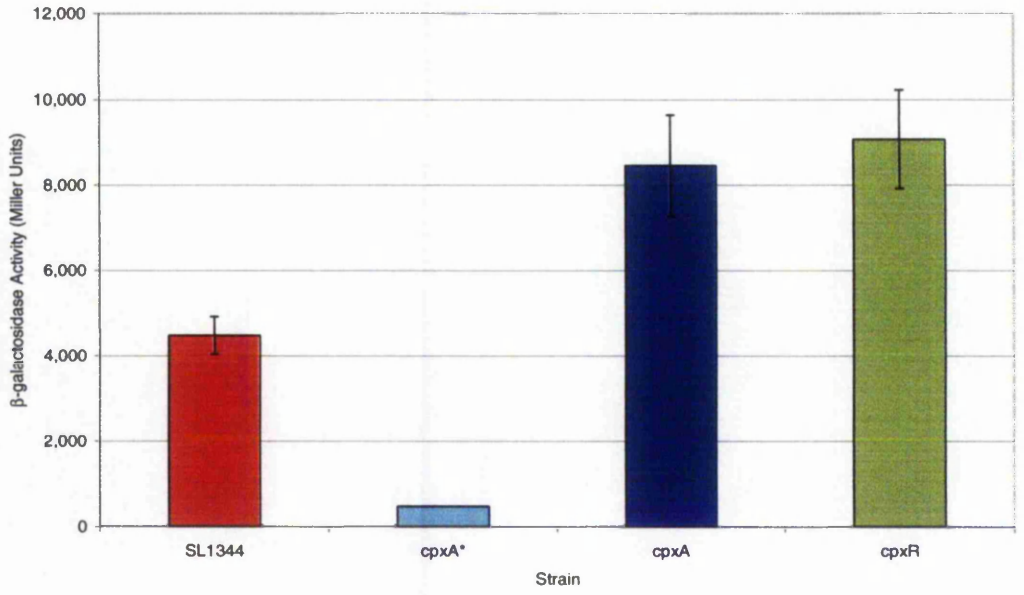
No affect on *cpxR* transcription was observed in *rpoN*, *htrA* or *surA* null mutant strains (GR, data not shown). The lack of affect of *surA* on *cpxRA* expression is further evidence for differential inducing cues for the σ^E and Cpx regulated responses.

4.7 Cross Talk Between the *S.* Typhimurium RpoE and Cpx Regulons

Figure 12A depicts activity of the *rpoE*P3 promoter in the various *cpx* mutant backgrounds previously described. We did this analysis to see if the *cpxRA* two component regulator of *S.* Typhimurium inhibited expression of the *rpoE* operon as described in *E. coli*. This appears to be the case, as activity of the *rpoE*P3 promoter in *S.* Typhimurium is inhibited in the constitutively active *cpxA** strain and enhanced in the *cpxA* and *cpxR* null mutant backgrounds relative to the wild type strain.

We were interested to see whether this cross talk between these regulatory pathways was uni- or bidirectional, and investigated this by analysing activity of the *cpxR* promoter in an *rpoE* mutant background. Figure 12B illustrates this data with a massive increase in *cpxRA* expression seen in the absence of functional σ^E. The expression levels seen in the absence of σ^E are some 40 times greater than that seen in the cpxA* background. However, activity of the *cpxR* promoter is not repressed after over-expression of σ^E.

A)



B)

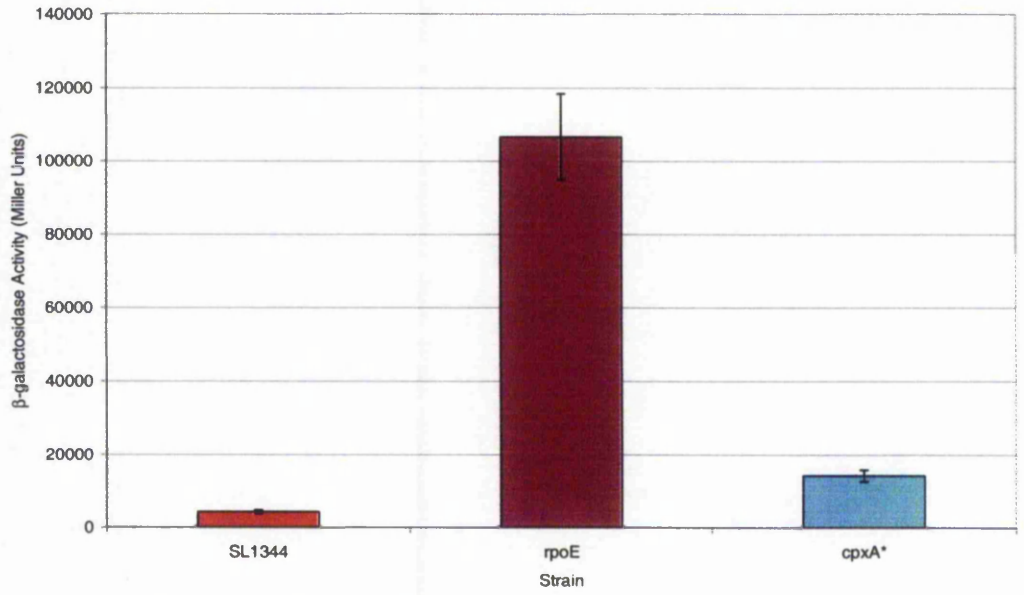


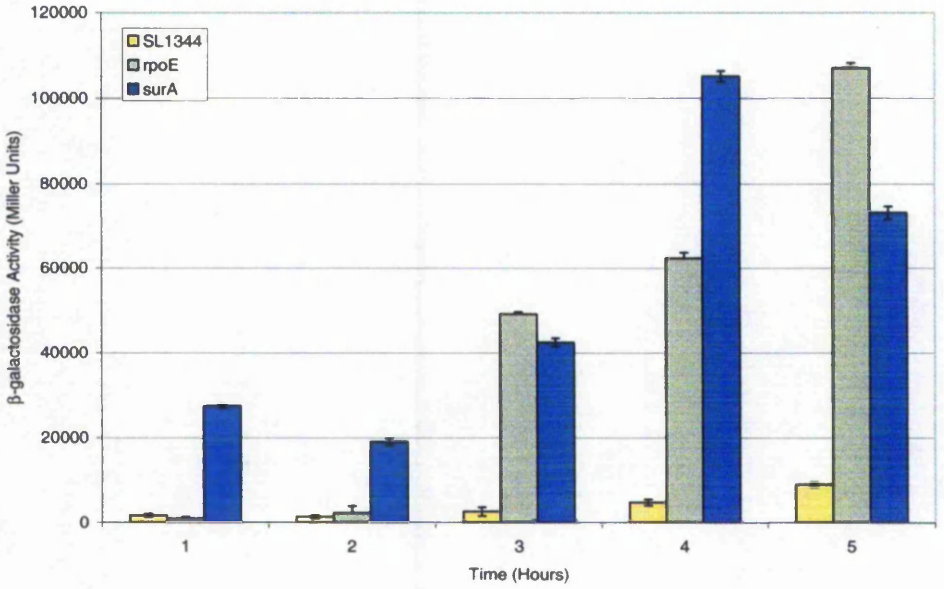
Figure 12. Evidence of cross talk between the ESR pathways.
A) depicts β galactosidase activity of the autoregulated *rpoEP3* promoter in the *cpx* null and constitutively active *cpx* mutants. **B)** depicts the equivalent experiment with activity of the *cpxR* promoter in an *rpoE* mutant strain illustrated.

4.8 RpoE Regulation of RpoH

Cells are believed to sense some forms of stress by monitoring the protein folding pathways within a cellular compartment, and predominantly these responses remain compartmentalised. In *E. coli* the σ^E and σ^H, ESR and cytoplasmic responses respectively, have been classified as compartmentalised responses. This classification is somewhat ambiguous as *rpoH* itself was one of the first σ^E regulated genes identified. We were keen to ascertain in *S.* Typhimurium whether regulation of *rpoH* was partly dependent on σ^E, as we hypothesised from the characterisation performed in *E. coli* that it would be. To do this we made a transcriptional fusion of the entire regulatory region of *rpoH* to *lacZ*, based on the promoter and enhancer regions predicted in *E. coli*, as illustrated in figure 6 and (Nagai *et al.*, 1990;Kallipolitis and Valentin-Hansen, 1998;Pallen, 1999;Ramirez-Santos *et al.*, 2001;Solis-Guzman *et al.*, 2001).

Figure 13 demonstrates both the effect of genetic backgrounds known to induce *rpoE* on the activity of the *rpoH* promoter region and the effect of an *rpoE* mutation on its activity. In wild type *S.* Typhimurium strain SL1344 there is a two fold increase in the promoter activity at the onset of stationary phase concurrent with stationary phase being an inducing condition of *rpoE.* This could be indicative of a σ^E regulated promoter being present, as could the massive increase in expression observed in a *surA* mutant background. In the absence of *rpoE*, from mid exponential phase onwards, the *rpoH* promoter is 10 fold more active than in the wild type strain.

A)



B)

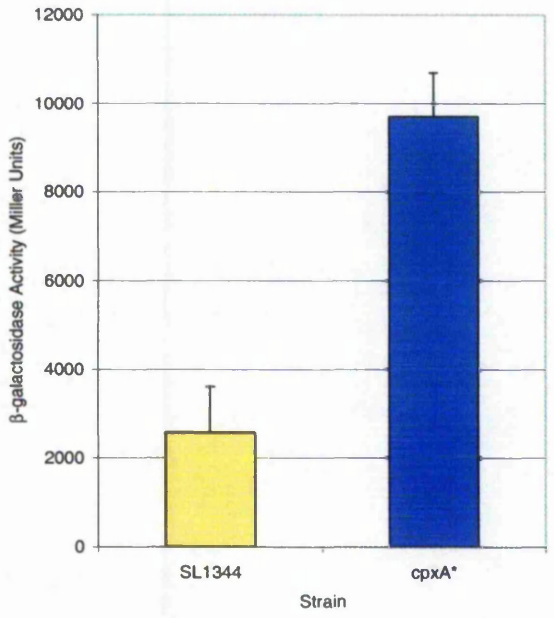
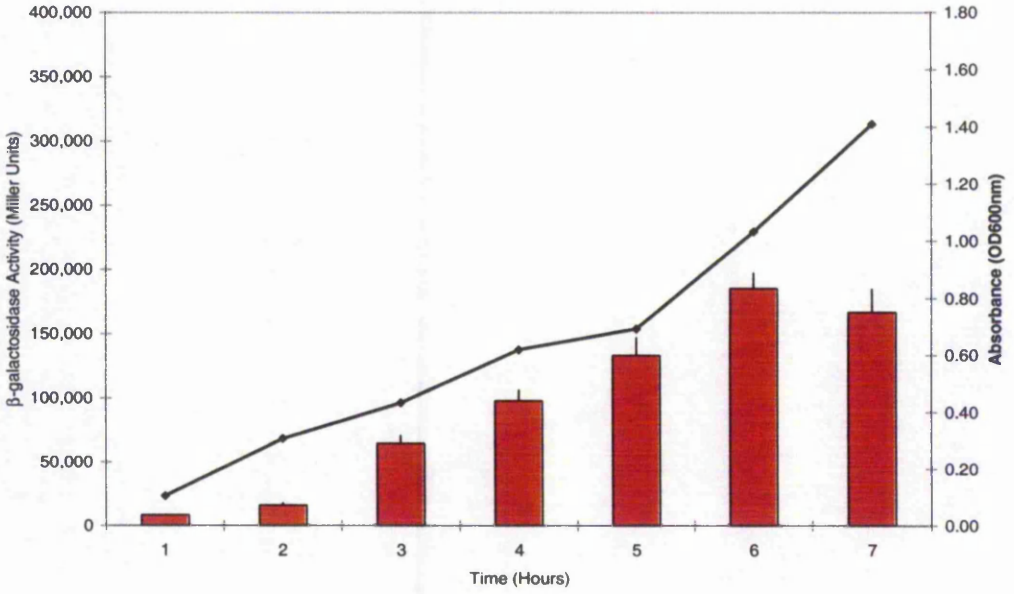


Figure 13. The absence of functional σ^E leads to induction of the cytoplasmic stress response.

A) Comparison of activity of the *rpoH* promoter construct in WT, *surA* and *rpoE* mutant strains of *S.* Typhimurium measured by β-galactosidase assay and **B)** in the constitutively active *cpxA** mutant background (mid log).

A)



B)

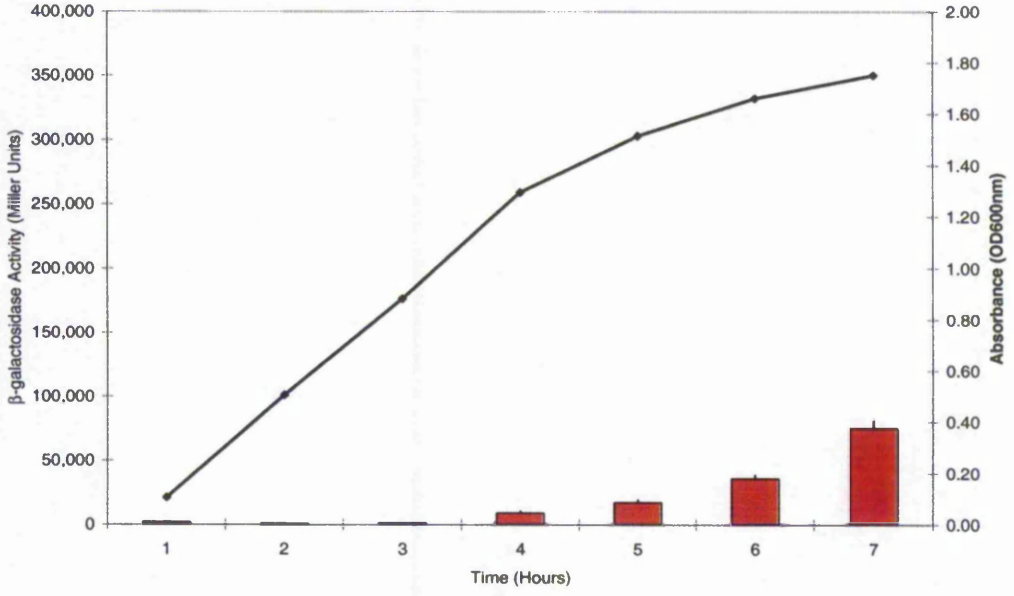


Figure 14. Effects of σ^E overexpression on activity of the *rpoH* regulatory region

The same experiment as described in figure 8 but this time in an *rpoE* mutant background harbouring p*rpoH*pall and pAC-*rpoE*ST4.

This may indicate a degree of compensatory function of σ^H for σ^E and possible transduction of periplasmic stress to the cytoplasmic space. This data agrees with the activity of the *rpoH* promoter in a *cpxA** background, which is 5 fold greater than in the wild type strain, where levels of *rpoE* mRNA are known to be inhibited. No affect on σ^H transcription was observed in *rpoS, rpoN, htrA, cpxA* or *fkpA* null mutant backgrounds, even though CpxR and RpoN binding boxes are predicted with the cloned sequence (GR, data not shown).

Confirmation of a σ^E regulated *rpoH* promoter was completed by overexpression of σ^E in an *rpoE* mutant background as described earlier. In the presence of arabinose, activity of the *rpoHpall* is 20 times higher than in the presence of glucose 4 hours after growth indicating the presence of a σ^E dependent promoter (figure 14). Preliminary data (GR) not shown here also implicates σ^E in the σ^H response to extreme temperatures.

4.9 Discussion

We hypothesised that there would be a difference in the regulation of stress responses encountered by *E. coli* and *S.* Typhimurium due to the differential environments that they encounter. The differences observed and reported in this chapter have been summarised in (Miticka *et al.*, 2003;Humphreys *et al.*, 2004).

In agreement with (De Wulf *et al.*, 2002) the *rpoE* operon of *S.* Typhimurium is negatively regulated by CpxR-P, with levels of transcription measured from the σ^E dependent *rpoEP3* promoter in a *cpxA** background similar to that seen in the *rpoE* mutant. The *rpoE*P3 promoter is essential for expression of *rpoE* under conditions that activate the σ^E pathway; therefore, constitutive activation of the Cpx system may render σ^E levels suboptimal *in vivo*. This might account for the reduced virulence of a *S.* Typhimurium *cpxA** mutant in a murine model of infection (Humphreys *et al.*, 2004).

We were interested if this transcriptional regulation between *cpx* and *rpoE* was bidirectional, and we are the first to observe that a *cpxR* promoter is highly active in the absence of functional σ^E, and even more active than in the constitutively active *cpxA** mutant. Overexpression of σ^E has no negative affect on activity of this promoter, so it seems unlikely that a member of the σ^E regulon acts as a negative regulator of the *cpxRA* pathway. What is more likely is the absence of *rpoE* results in instability of the periplasmic environment leading to activation of the *cpxRA* pathway.

Involvement of the alternative sigma factor, σ^S, has been proposed for regulation of the *cpxRA* pathway of *E. coli* (De Wulf *et al.*, 2002). No difference in activity of either the *prpoEP3* or *pcpxRs* promoters was observed between the wild type strain or an *rpoS* mutant, indicating no overlap between the ESR and RpoS mediated response in *S.* Typhimurium, at least at the transcriptional level. However this should be confirmed by qRT-PCR or Northern blot as the RpoS regulated promoter may lay outwith the regulatory regions used in the reporter plasmids.

Although the periplasmic and cytoplasmic stress responses have been described as compartmentalised, one wonders if this is indeed the case, as in *E. coli* and now in *S.* Typhimurium σ^H is itself regulated by σ^E. The data presented in this chapter may also further suggest that there is a degree of fluidity between the two stress responses, with activity of the σ^H promoter construct markedly increased in the absence of σ^E. This is an area for further research, to investigate whether periplasmic stress is transduced across the cytoplasmic membrane to induce the cytoplasmic stress response and also to search for genes that are co-regulated by both σ^E and σ^H.

Induction of the *htrA* promoter in mammalian cells encouraged us to investigate the activity of the *rpoE* promoters in a similar situation. Induction of both *prpoEP1* and *prpoEP3* was observed between 4 and 24 hours post infection, although the highest

transcription levels were measured with *prpoEP3*. Our explanation for the induction of *prpoEP1* is through σ^E mediated upregulation of *rpoD* transcription. We predict that the autoregulated *rpoE*P3 promoter could potentially be suitable for expression of heterologous antigens in *Salmonella*.

This aspect of the study attempted to address a number of questions regarding regulation of the ESR in *S.* Typhimurium. However a number of avenues and questions for future investigation have been opened. Confirmation of the work performed here using transcriptional fusions should be confirmed via quantitative PCR to ensure that expression levels relating to promoter activity correlate with mRNA transcript levels, although some of the analysis has been confirmed from the micorarray data. This work could also be adapted to investigate any overlap in post translational regulation of the ESR.

Investigation into activity of the *cpxR* and *rpoH* promoters should also be analysed in mammalian cells, and one would predict that the *rpoH* promoter would also be induced by the intracellular environment due to regulation of this gene by σ^E, and due to its upregulation observed in macrophages via microarrays (Eriksson *et al.*, 2003). The *rpoE* and *rpoH* promoters could also be used to try and express heterologous antigens such as the tetanus protective antigen, fragment C, in vaccine strains of *Salmonella*. We have used the reporter constructs in this study to investigate inducing conditions of the ESR stress responses. This work should be continued further to investigate a wide range of potential inducing cues, and fully characterise the environmental signals involved in stimulation of the ESR and fully elucidate the differences between the ESR of *S.* Typhimurium and *E. coli*.

Chapter 5 - Hunting for RpoE Regulated Genes in *S.* Typhimurium

5.1 Introduction

At the beginning of this study our knowledge of the ESR of *S.* Typhimurium was limited. We were aware that an *S.* Typhimurium *rpoE* mutant was viable, unlike in *E. coli*, and that it possessed several phenotypes of interest, especially the severity of attenuation observed in a murine model of infection. However our understanding of genes that were σ^E regulated was governed by research in *E. coli.*

To identify σ^E regulated genes in *E. coli* a number of methods have been employed, all of which have identified different regulon members (Dartigalongue, Missiakas, and Raina, 2001) (Rezuchova *et al.*, 2003). With this in mind we were reluctant to rely on a single method to identify σ^E regulated genes in *S.* Typhimurium. We therefore devised a three pronged approach, which involved several extremely useful and successful collaborations. The first collaboration established was with the group of Dr Jan Kormanec, at the Slovak Academy of Science, Slovak Republic. This group had already developed a two plasmid technique (described below) to identify genes regulated by the sigma factor RpoZ in Streptomyces aureofaciens (Novakova, Sevcikova, and Kormanec, 1998), and were in the progress of using this same technique to identify σ^E regulated genes in *E. coli* (Rezuchova and Kormanec, 2001). The Kormanec group would identify putatively σ^E regulated genes using this system and then further confirm this regulation by using S1 end nuclease mapping (Kormanec, 2001). We would then use this knowledge to make chromosomal deletions of the putative genes and try and assess their role both *in vitro* and *in vivo.*

The second prong of our approach utilised microarray technology and was performed in collaboration with Dr. Jay Hinton's group at the Institute of Food Research (IFR), Norwich. The decision to use microarray technology was made on the grounds that the

two plasmid system was extremely labour intensive and although successful in identifying putatively σ^E regulated genes in *S.* Typhimurium, relying solely on this method would mean slow progress for this study. Initially our aim was to extract total RNA from stationary phase cultures of wild type SL1344 and the *rpoE* mutant, as stationary phase growth was one of the few confirmed inducing conditions of σ^E expression at this time. However, the quality of RNA extracted from the *rpoE* mutant under these conditions was extremely poor. To overcome this problem we compared total RNA from wild type SL1344 overexpressing σ^E under the control of an arabinose responsive promoter, to the same strain containing only empty vector. This RNA was then labelled at the IFR and hybridised onto *S.* Typhimurium LT2a arrays. The Hinton group have successfully used their arrays to identify *S.* Typhimurium genes involved in macrophage infection (Eriksson *et al.*, 2003) and the role of PNPase in persistent infection (Eriksson *et al.*, 2003), as well as most recently in analysis of the Fis regulon (Kelly *et al.*, 2004).

The third prong of our approach utilised 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. The screen was performed using degenerative nucleotide strings in Artemis (Rutherford *et al.*, 2000) and mismatch pattern searches in colibase (Chaudhuri, Khan, and Pallen, 2004). The importance of certain nucleotides in the -10 and -35 regions for activity of the *rpoEP3* promoter have now been elucidated (Miticka *et al.*, 2004) and this was also taken into account when deciding upon the probe sequence used for the searches.

The genes identified in these screens are at this stage mostly still putatively σ^E regulated. Due to the nature of our microarrays it is possible that upregulated genes

could be controlled by other regulatory factors such as σ^H, due to the presence of a σ^E regulated promoter upstream of the *rpoH* gene. However, we are particularly confident in genes that have been identified by more than one of our screens especially those that have a good promoter consensus or have a putative promoter missing in an *S.* Typhimurium *rpoE* mutant after S1 nuclease mapping.

5.2 The Two Plasmid Screen

The two plasmid screen involves using a single bacterial strain hosting one plasmid containing the *rpoE* gene under the control of an arabinose inducible promoter (pAC-*rpoE*ST4) and a second plasmid containing a fragment of *S.* Typhimurium chromosome DNA upstream of a promoterless *lacZ* gene, generated from a shotgun library. When plated onto solid media, supplemented with arabinose and Xgal, any blue colonies indicate that potentially the cloned chromosomal fragment contains a σ^E dependent promoter. This promoter can be confirmed by sequencing, S1 mapping and primer extension. Using this method the Kormanec group identified 32 putatively σ^E regulated promoters, only eight of which at the time had been identified in *E. coli* (Dartigalongue, Missiakas, and Raina, 2001). These genes are all listed in table 5. Subsequently another 3 of these genes were also identified in the two plasmid screen for σ^E regulated genes in *E. coli* (Rezuchova *et al.*, 2003).

Gene name	Function
	Transcriptional factors and Regulatory genes
rpoE (*stm2640*)	σ^E/ECF sigma factor
rpoH (*stm3568*)	σ^{32} /classical heat shock factor
rpoD (*stm3211*)	σ^{70}/housekeeping sigma factor
	Primary metabolism functions
fusA (*stm3446*)	Translation EF-G, EF-Tu
eno (*stm2952*)	Enolase (glycolysis)
mlc (*stm1488*)	NagC-like transcriptional regulator, global
ybbN (*stm0504*)	Thioredoxin-like protein
ptr (*stm2995*)	Protease III, exonuclease V subunits
	Periplasmic folding factors
htrA (*stm0209*)	Periplasmic serine protease
Stm1250	Putative molecular chaperone
stm 1251(agsA)	Putative molecular chaperone
fkpA (*stm3453*)	Peptidyl-prolyl-cis-trans-isomerase
surA (*stm0092*)	Peptidyl-prolyl-cis-trans-isomerase
	Membrane or Phospholipid biogenesis
plsB (*stm4235*)	Glycerol-3-phosphate acyltransferase
psd (*stm4348*)	Phosphatidylserine decarboxylase
	Unknown functions
yggT (*stm3101*)	Integral membrane resistance protein
b1973 (*stm1263*)	Periplasmic protein
bax (*stm3663*)	ATP-binding protein
yjfO (*stm4379*)	Lipoprotein
yfiO (*stm2663,ecfD*)	Lipoprotein
dedD (*stm2364*)	Lipoprotein
sbmA (*stm0376*)	ABC superfamily transporter
yraP (*stm3267,ecfH*)	Periplasmic protein.
ygiM (*stm3203,ecfG*)	SH3 domain protein,
tolR (*stm0746*)	IMP, outer membrane integrity
ydcG (*stm1622*)	periplasmic glucans biosynthesis protein
yccV (*stm1079*)	IMP
ycbK (*stm0997*)	OMP, metallo-beta-lactamase
yabI (*stm0105*)	DedA family membrane protein
yiiD (*stm4029*)	Acetyltransferase
yrfH (*stm3497*)	Heat-shock protein
yfeK (*stm2438*)	Periplasmic protein, penicillin-binding

Table 5 – A list of putative *S.* Typhimurium σ^E regulated genes identified by the two plasmid screen. The genes have been functionally grouped, but interestingly the majority are still of unknown function. Where genes are located in operons only the first gene of the operon is listed.

5.3 IFR Microarray Analysis

Microarray analysis in this thesis was used as a tool to try and identify candidate σ^E regulated genes for further investigation.

The RNA was extracted as per Materials and Methods. This was reverse-transcribed, labelled with Cy5-dCTP and co-hybridised with LT2 genomic DNA labelled with Cy3-dCTP to microarray slides containing 4418 open reading frames (ORFs). This represents 97% of the *S.* Typhimurium LT2 genes, and was completed at The Institute of Food Research, Norwich. Duplicate RNA samples from each culture were hybridised to 2 arrays (*i.e.* 4 arrays per strain). After hybridisation, slides were washed twice for 10 minutes in each of the following successive washes: 2X SSC, 0.1% SDS at 65°C; 1X SSC at room temperature and 0.2X SSC at room temperature. The slides were dried by centrifugation and scanned on a GenePix 4000A scanner (Axon Instruments). The fluorescent intensities for each cDNA/genomic DNA spot were measured using GenePix Pro 3.0 software (Axon Instruments). All spots with a fluorescent intensity of less than 2 standard deviations above background were excluded from further analysis. Inequalities in dye incorporation or template concentration were compensated by data centering, bringing the natural logarithm of the ratios for each group of spots printed by the same pin to zero. Data passing the quality controls was analysed using GeneSpring 6.1 software (Silicon Genetics). Significance of the data at $P<0.05$ was measured using a parametric-based test, adjusting the individual *P*-value with the Benjamini and Hochberg false-discovery rate multiple test correction. The data presented here is for a single biological replicate, and can be viewed in it's entirety as an appendix to this thesis.

Table 6 is a list all of the genes whose expression was significantly upregulated ($p<0.05$) greater than 1.5 fold in the strain overexpressing σ^E (pAC-rpoEST4). Data was normalised to expression levels obtained from the *S.* Typhimurium strain containing only the empty vector (pAC7). In total 438 genes were significantly upregulated more than 1.5 fold which is approximately 7% of the *S.* Typhimurium genome. If a greater degree of stringency is applied 184 genes are significantly upregulated greater than two fold and 70 genes greater than three fold. Only 11 genes were significantly repressed, less than 0.5 fold. Encouragingly the top three genes with highest fold induction were all already known to be σ^E regulated according to *E. coli*, with the gene resulting in the highest normalised ratio being *rpoE* itself. This was expected due to the nature of our overexpression technique. This is depicted in figure 15.

The requirement for functional *rpoE* for cell viability is something that has been discussed in recent times with *E. coli* K12 σ^E required for survival where as in *S.* Typhimurium, and now in several other bacteria, it is not. However, the increased lag phase of the σ^E mutant in the absence of glucose as a carbon source does suggest a degree of growth impairment. With this in mind we searched the 257 genes that had been described as essential for *S.* Typhimurium viability (Knuth *et al.*, 2004) for genes that were putatively σ^E regulated. Table 7 lists the 60 genes classified as indispensable for *S.* Typhimurium growth which were upregulated greater than 1.5 fold after over-expression of σ^E. However this should be treated with caution, as after further examination of these so called essential genes, one of them is *surA*, a gene that is already known to be σ^E regulated and has been successfully mutated in *S.* Typhimurium (Sydenham *et al.*, 2000).

Due to the degree of attenuation observed with a *S.* Typhimurium *rpoE* mutant in a murine model of infection, we began to look for potentially σ^E regulated genes that

were involved in virulence. Primary analysis looked for RpoE regulation of genes encoded by the *Salmonella* pathogenicity islands. Of the 438 genes significantly upregulated greater than 1.5 fold, none of the genes were members of the five pathogenicity islands. However 50 of the genes upregulated greater than 1.5 fold after overexpression of σ^E were also upregulated within J774 macrophages (Eriksson *et al.*, 2003). These include the σ^E operon itself and confirmed σ^E regulated genes in *E. coli* such as *htrA* and *rpoH*. Other genes not identified in *E. coli* that fall into this subset of genes include the phage shock response (*psp*) operon, *yiaD*, *bacA*, and the *Salmonella* specific gene stm1251 (*agsA*).

The other subset of genes to note is those that are function unknown (FUN) (Hinton, 1997). Genes of unknown function are considered to be potential antibiotic and vaccine targets. Fifty one of the 438 genes significantly upregulated greater than 1.5 fold after overexpression of σ^E are of unknown function. This equates to approximately 12% of the upregulated genes identified by array analysis, and is indicative of the level of resolution still required to even begin to understand the *S.* Typhimurium *rpoE* regulon.

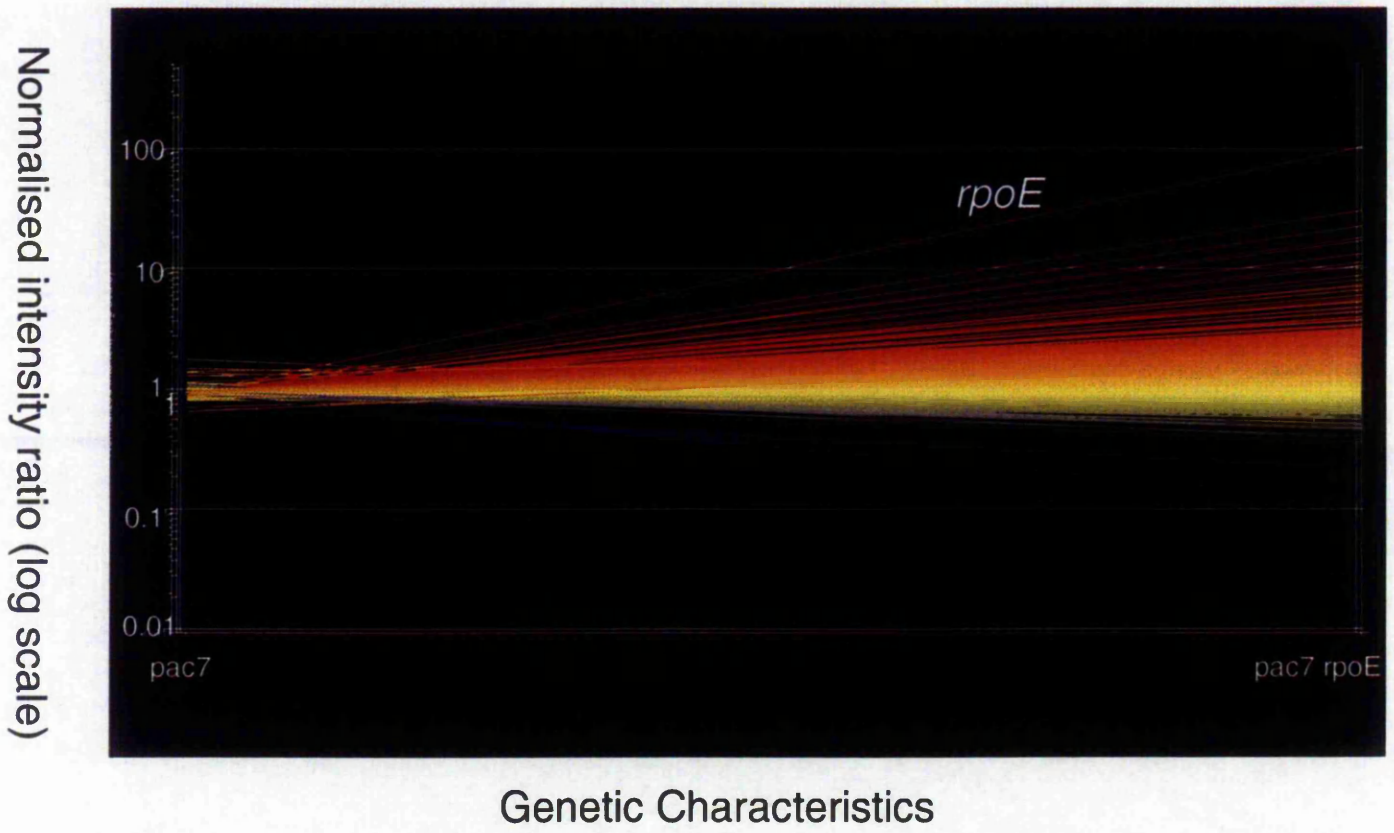


Figure 15. A genespring (Silicon Genetics) image depicting the *S.* Typhimurium array dataset after overexpression of σ^E.
Data is coloured by expression levels and normalised to the strain carrying empty vector (pac7). Red lines are indicative of upregulation of expression and blue indicative of repression. The gene with the greatest level of upregulation was *rpoE*.

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM2640	rpoE	111.62831	1.67E-11	sigma E (sigma 24) factor
STM0209	htrA	32.751595	1.77E-06	periplasmic serine protease
STM2639	rseA	24.29473	1.05E-10	anti sigma E (sigma 24) factor
STM3645	yiaD	19.420488	4.23E-11	putative outer membrane lipoprotein
STM3267	yraP	18.33302	3.36E-09	paral putative periplasmic protein
STM1251		14.328271	6.58E-11	putative molecular chaperone
STM2447		12.113163	5.68E-11	putative outer membrane lipoprotein
STM1250		11.348843	3.67E-10	hypothetical protein
STM1746	oppA	11.31134	1.30E-12	ABC superfamily (periplasm),
STM3107	yggN	9.500106	2.18E-09	hypothetical protein
STM0449	clpX	8.756819	1.90E-06	ATP-dependent serine protease
STM2488	nlpB	7.910659	2.35E-11	lipoprotein-34
STM2663	yfiO	7.8763113	1.67E-05	putative lipoprotein
STM2685	smpA	7.703394	9.97E-09	small membrane protein A
STM3568	rpoH	7.1996913	3.38E-10	sigma H (sigma 32) factor
STM2364	dedD	7.1878834	1.10E-08	putative lipoprotein
STM3203	ygiM	7.180906	8.08E-11	hypothetical protein
STM0225	skp	7.1352363	1.13E-09	histone-like protein
STM3808	ibpB	7.0378857	7.82E-08	small heat shock protein
STM3809	ibpA	6.5722375	8.81E-11	small heat shock protein
STM1819	slp	6.109265	4.60E-09	putative outer membrane protein
STM0092	surA	5.9183993	1.69E-11	peptidyl-prolyl cis-trans isomerase
STM3967	dlhH	5.829285	1.06E-08	putative dienelactone hydrolase family
STM2401	ddg	5.809243	4.26E-09	cold shock-induced palmitoleoyl transferase

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM0224	yaeT	5.782642	2.82E-10	putative outer membrane antigen
STM1150	mdoG	5.701717	2.27E-09	periplasmic glucans biosynthesis protein
STM3211	rpoD	5.1566143	1.87E-08	sigma D (sigma 70) factor
STM4348	psd	5.1048446	7.61E-10	phosphatidylserine decarboxylase
STM2995	ptr	4.7431006	2.12E-08	protease III
STM0133	ftsZ	4.740666	1.32E-08	tubulin-like GTP-binding protein
STM1782	ychH	4.6614203	6.33E-10	putative inner membrane protein
STM2521	yfgM	4.6425786	2.66E-08	putative inner membrane protein
STM1344	ydiV	4.64254	3.65E-10	putative diguanylate cyclase
STM0746	tolR	4.299367	5.33E-10	tol protein
STM2638	rseB	4.2888627	1.97E-08	anti sigma E (sigma 24) factor
STM1747		4.268536	1.38E-08	putative inner membrane protein
STM2387	sixA	4.253839	3.74E-07	phosphohistidine phosphatase
STM1253		4.2504306	8.01E-07	putative inner membrane protein Cytochrome B561
STM0814	ybhQ	4.2479677	1.55E-07	putative inner membrane protein
STM3321	yhbH	4.0547624	2.83E-04	probable sigma N modulation factor
STM4235	plsB	4.0077977	7.73E-08	glycerolphosphate acyltransferase activity
STM2495	yfgD	4.0011573	1.56E-08	putative arsenate reductase
STM0226	lpxD	3.991088	7.58E-09	UDP-3-O-(3-hydroxymyristoyl)-glucosamine n-acyltransferase
STM0376	sbmA	3.937798	1.56E-06	putative ABC superfamily transporter
STM3374	mreB	3.7596009	2.29E-08	rod shape-determining protein
STM0105	yabI	3.6863642	9.81E-09	membrane-associated protein
STM3101	yggT	3.6835637	1.10E-08	putative integral membrane resistance protein
STM1324		3.6042528	0.017707	putative cytoplasmic protein

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM0223	yaeL	3.5893304	4.29E-09	predicted membrane-associated Zn-dependent protease
STM1690	pspA	3.4374547	1.84E-08	phage shock protein
STM3448	rpsL	3.4355953	4.04E-07	30S ribosomal subunit protein S12
STM2520	sspJ	3.3803174	2.10E-08	putative serine/threonine protein kinase
STM0012	dnaK	3.3463604	4.71E-08	chaperone Hsp70
STM0413	tsx	3.3172643	3.15E-08	nucleoside channel
STM2558	cadB	3.311247	1.93E-08	APC family, lysine/cadaverine transport protein
STM1743	oppD	3.29727	1.04E-08	ABC superfamily (atp-binding), oligopeptide transport protein
STM3452	yheO	3.2156255	1.53E-08	putative regulator
STM4330	groEL	3.1711426	1.27E-07	chaperone Hsp60
STM3420	secY	3.121317	2.38E-07	preprotein translocase of IISP family
STM1151	mdoH	3.1206465	7.46E-09	membrane glycosyltransferase
STM1744	oppC	3.0951078	2.87E-09	ABC superfamily (membrane), oligopeptide transport protein (2nd module)
STM3447	rpsG	3.0906477	9.75E-07	30S ribosomal subunit protein S7
STM1745	oppB	3.0837367	4.57E-09	ABC superfamily (membrane), oligopeptide transport protein
STM3635	yhjW	3.0410588	3.46E-08	predicted membrane-associated
STM3446	fusA	3.0340471	1.74E-06	protein chain elongation factor EF-G
STM3428	rplE	3.0157654	2.12E-08	50S ribosomal subunit protein L5
STM3016	area	3.0112143	1.07E-08	proton symport protein
STM1286	mipA	2.9948246	1.36E-07	scaffolding protein for murein-synthesizing holoenzyme
STM2494		2.993048	1.42E-08	putative inner membrane
STM3417	rpsK	2.9433205	1.25E-08	30S ribosomal subunit protein S11
STM2489	dapA	2.9283218	0.015958	dihydrodipicolinate synthase
STM0377	yaiW	2.9140794	4.14E-08	hypothetical protein

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM1742	oppF	2.8938138	7.53E-07	ABC superfamily (atp-binding), oligopeptide transport protein
STM0043	rpsT	2.8936608	4.43E-04	30S ribosomal subunit protein S20
PSLT103	traT	2.8934429	2.28E-08	*S.* Typhimurium 90 kb virulence plasmid complement resistance protein
STM3437	rplB	2.8710217	1.15E-06	50S ribosomal subunit protein L2
STM1064	pqiB	2.8566778	4.71E-06	paraquat-inducible protein B (1st module)
STM4304	dcuS	2.854595	4.79E-04	sensory histidine kinase in two component regulatory system with DcuR
STM3103	yggV	2.8526201	1.22E-07	putative xanthosine triphosphate
STM0997	ycbL	2.8524914	2.63E-08	metallo-beta-lactamase
STM2327	nuoB	2.840095	1.72E-06	NADH dehydrogenase I chain B
PSLT102	traS	2.8229666	2.10E-08	surface exclusion protein
STM3434	rpsC	2.8153605	2.91E-07	30S ribosomal subunit protein S3
STM4128	yijD	2.8128653	3.91E-08	orf
STM0106	yabJ	2.811958	8.04E-06	hypothetical ABC-transport protein
STM0441	cyoC	2.8046055	1.92E-05	cytochrome o ubiquinol oxidase subunit III
STM0749	pal	2.7939184	5.76E-07	tol protein required for outer membrane integrity
STM0748	tolB	2.779544	8.35E-07	tol protein required for outer membrane integrity
STM2676	rpsP	2.7680185	2.56E-06	30S ribosomal subunit protein S16
STM3322	ptsN	2.7434404	9.17E-08	sugar specific PTS family
STM1938	yecA	2.7426274	6.06E-04	hypothetical protein
STM4029	yiiD	2.6838932	6.97E-06	conserved hypothetical protein
STM3429	rplX	2.663269	2.28E-08	50S ribosomal subunit protein L24
STM3866	atpG	2.6617231	8.91E-08	membrane-bound ATP synthase
STM0750	ybgF	2.6537015	1.52E-07	putative periplasmic protein
STM3425	rplF	2.6111865	1.15E-06	50S ribosomal subunit protein L6

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM3741	rpoZ	2.6066115	3.27E-08	RNA polymerase, omega subunit
STM3867	atpA	2.6058805	1.51E-08	membrane-bound ATP synthase , F1 sector, alpha-subunit(2nd module)
STM3319	yhbG	2.604473	8.07E-07	putative ABC superfamily (ATP binding) transport protein
STM3436	rpsS	2.5876698	1.16E-06	30S ribosomal subunit protein S19
STM3732	ttk	2.5852728	0.048995	putative transcriptional regulator (TetR/ArcR family)
STM1688	pspC	2.561426	1.05E-06	phage shock protein; regulatory gene
STM4150	rplA	2.552002	3.73E-07	50S ribosomal subunit protein L1
PSLT067		2.539842	1.91E-07	putative cytoplasmic protein
STM2675	rimM	2.534206	5.85E-07	16S rRNA processing protein
STM3968	udp	2.5309803	2.50E-04	uridine phosphorylase
STM1161	yceP	2.5164871	1.60E-06	putative cytoplasmic protein
STM4347	yjeP	2.507968	1.02E-06	putative periplasmic binding protein (2nd module)
STM0132	ftsA	2.4700844	5.47E-07	ATP-binding cell division protein
STM4097		2.4579794	1.57E-07	putative outer membrane lipoprotein
STM0733	sdhD	2.443882	2.06E-07	succinate dehydrogenase
STM3296	hflB	2.4316096	2.67E-06	ATP-dependent zinc-metallo protease (2nd module)
STM0216	rpsB	2.4247224	6.97E-07	30S ribosomal subunit protein S2
PSLT104	traD	2.3913538	9.16E-07	*S.* Typhimurium F-related plasmid protein
STM0227	fabZ	2.3871548	1.81E-07	(3R)-hydroxymyristol acyl carrier protein dehydratase
STM1479	pntA	2.38144	8.44E-07	pyridine nucleotide transhydrogenase (proton pump)
STM4588	creB	2.375637	2.64E-07	response regulator in two component regulatory system with CreC
STM0448	clpP	2.3740828	1.41E-06	ATP-dependent serine protease
STM1689	pspB	2.3483496	8.64E-07	phage shock protein; regulatory gene,
STM4329	mopB	2.3433316	7.53E-06	chaperone Hsp10, affects cell division

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM2432	ptsI	2.3401465	2.11E-07	General PTS family (Enzyme I)
STM1583		2.336867	7.76E-08	putative cytoplasmic protein
STM3473	yhfC	2.334601	6.47E-07	putative MFS family transport protein
STM3268	yraR	2.332758	2.74E-06	putative nucleoside-diphosphate-sugar epimerase
STM3450	yheM	2.3020544	1.74E-06	putative oxidation of intracellular sulfur
STM0607	dsbG	2.2907221	2.15E-07	periplasmic disulfide isomerase
PSLT041	spvR	2.2861416	2.37E-08	*S.* Typhimurium 9 Kb virulence transcriptional activator
STM3318	yhbN	2.2781556	1.36E-06	putative ABC superfamily transport protein
STM0228	lpxA	2.2653286	3.84E-07	UDP-N-acetylglucosamine acetyltransferase
STM1844	htpX	2.2581406	2.52E-06	heat shock protein, integral membrane protein
STM0049	lytB	2.2547414	9.39E-07	regulates the activity of guanosine 3',5'-bispyrophosphate synthetase I (RelA)
STM3427	rpsN	2.2542422	2.64E-06	30S ribosomal subunit protein S14
STM3861	glmS	2.23689	1.49E-06	L-glutamine:D-fructose-6-phosphate aminotransferase (1^{st} module)
STM3451	yheN	2.2055736	1.75E-06	hypothetical protein
STM0090	ksgA	2.2039785	5.57E-07	S-adenosylmethionine-6-N',N'-adenosyl (rRNA) dimethyltransferase
STM3430	rplN	2.198246	3.65E-07	50S ribosomal subunit protein L14
STM3204	cca	2.194532	2.30E-07	tRNA nucleotidyl transferase
STM3424	rplR	2.1930358	1.86E-04	50S ribosomal subunit protein L18
STM4587	creA	2.1916845	2.39E-07	hypothetical protein
STM0101	araD	2.1893346	2.40E-06	L-ribulose-5-phosphate 4-epimerase (1st module)
STM4272		2.1734934	0.215642	putative inner membrane protein
STM3865	atpD	2.1733532	1.55E-06	membrane-bound ATP synthase
STM2660	clpB	2.1650376	9.17E-07	ATP-dependent protease, Hsp 100
STM2325	nuoE	2.1635187	2.82E-06	NADH dehydrogenase I chain E

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM4349	yjeQ	2.1632364	3.10E-07	predicted GTPase
STM3868	atpH	2.1481633	1.88E-05	membrane-bound ATP synthase
STM2781	virK	2.1295397	3.59E-06	virulence gene
STM4363	hflK	2.1253464	2.70E-06	with HflC, part of modulator for protease specific for FtsH phage lambda cII repressor
STM3613	yhjJ	2.122231	2.24E-06	predicted Zn-dependent peptidase
STM0048	slpA	2.1174943	6.39E-07	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)
STM3297	ftsJ	2.116702	4.69E-06	23S rRNA methyltransferase
STM4391	rpsF	2.1135333	5.37E-07	30S ribosomal subunit protein S6
PSLT053	parB	2.1105824	3.18E-07	58% identity with *E. coli* plasmid P1 partition protein (PARB) (SW:P07621)
STM2496	yfgE	2.1095378	7.84E-07	putative ATPase involved in DNA replication initiation
STM2438	yfeK	2.1093822	3.87E-07	hypothetical protein
STM2386	yfcN	2.0998893	1.31E-06	hypothetical protein
STM2771	fljB	2.0765145	2.91E-06	Flagellar synthesis: phase 2 flagellin (filament structural protein)
STM3323	yhbJ	2.0753083	1.35E-06	hypothetical protein
STM3439	rplD	2.0704348	4.31E-05	50S ribosomal subunit protein L4, regulates expression of S10 operon
STM4362	hflX	2.0655422	1.41E-05	putative GTP-ase
PSLT101	traG	2.0623448	1.08E-05	86% identity with *E. coli* plasmid F protein (TRAG) (SW:P33790)
STM0442	cyoB	2.0565822	3.75E-06	cytochrome o ubiquinol oxidase subunit I
STM1879	ptrB	2.0515172	0.064191	protease II
STM0443	cyoA	2.0448353	3.53E-06	cytochrome o ubiquinol oxidase subunit II
STM1686	pspE	2.038051	6.00E-07	phage shock protein
PSLT042		2.03174	7.23E-07	100% identity with S. choleraesuis protein (M5)
STM3422	rpmD	2.0280719	7.79E-06	50S ribosomal subunit protein L30
STM0153	aceF	2.0262918	9.99E-06	pyruvate dehydrogenase

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM3205	bacA	2.0224133	6.00E-07	bacitracin resistance; possibly phosphorylates undecaprenol
STM1622	ydcG	2.0031617	5.18E-06	paral putative periplasmic glucans biosynthesis protein
STM2993	recD	2.0023901	2.48E-06	exonuclease V, alpha chain
STM2970	sdaC	2.0022645	1.21E-06	putative HAAAP family, serine transport protein
STM3419	rpmJ	1.9878412	1.11E-06	50S ribosomal subunit protein X
STM3870	atpE	1.9852182	3.96E-06	membrane-bound ATP synthase, F0 sector, subunit c
STM1249		1.9846722	9.67E-07	hypothetical protein
STM1203	ptsG	1.9814234	1.49E-06	Sugar Specific PTS family,
STM2324	nuoF	1.9779457	1.09E-06	NADH dehydrogenase I chain F (1st module)
STM2267	ompC	1.9742663	2.52E-05	outer membrane protein 1b (ib;c), porin (2nd module)
STM2326	nuoC	1.9634567	4.65E-06	NADH dehydrogenase I chain C,D
STM2637	rseC	1.9552509	3.39E-06	regulator of sigma E (sigma 24) factor
STM2662	rluD	1.9486134	1.22E-06	pseudouridine synthase (pseudouridines 1911, 1915, 1917 in 23S RNA)
STM0732	sdhC	1.946517	1.09E-06	succinate dehydrogenase , cytochrome b556
STM1063	pqiA	1.9438367	2.21E-06	paraquat-inducible protein A
STM2674	trmD	1.9387122	1.73E-04	tRNA (guanine-7-)-methyltransferase
STM1214	ycfR	1.9308331	3.48E-05	hypothetical protein
STM3281	nlpI	1.9288867	3.79E-05	lipoprotein, cell division
STM1065	ymbA	1.9268963	5.71E-06	hypothetical protein NB adjacent to pqiA and pqiB
STM1195	fabG	1.9239112	7.39E-06	3-oxoacyl-[acyl-carrier-protein] reductase
STM3487	aroK	1.914732	8.49E-06	shikimate kinase I
STM4569	deoB	1.9114492	4.37E-06	phosphopentomutase
STM2879	sicP	1.9078869	1.60E-06	chaparone, related to virulence
STM0440	cyoD	1.9078064	1.76E-06	cytochrome o ubiquinol oxidase subunit IV

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM1193	fabH	1.9046866	3.81E-04	3-oxoacyl-[acyl-carrier-protein] synthase III; acetylCoA ACP transacylase
STM1687	pspD	1.9021288	2.44E-06	phage shock protein
STM3298	yhbY	1.893833	1.52E-05	predicted RNA-binding protein containing KH domain
STM1682	tpx	1.8916097	7.11E-06	thiol peroxidase
STM1334	infC	1.8900976	8.83E-06	protein chain initiation factor IF-3
STM0217	tsf	1.8884375	7.53E-06	protein chain elongation factor EF-Ts
STM3423	rpsE	1.8812624	9.91E-06	30S ribosomal subunit protein S5
STM3416	rpsD	1.8797288	4.13E-06	30S ribosomal subunit protein S4
STM4091	hslU	1.8769081	6.82E-05	ATPase component of the HslUV protease (1st module)
STM2487	purC	1.875529	8.30E-06	phosphoribosylaminoimidazole-succinocarboxamide synthetase (SAICAR synthetase)
STM3320	rpoN	1.8747576	2.32E-05	sigma N (sigma 54) factor of RNA polymerase
STM4378	yjfN	1.8690915	2.82E-06	putative inner membrane protein
STM2782	mig-14	1.8650233	6.23E-06	putative transcription activator
PSLT025		1.8621306	1.88E-04	putative cytoplasmic protein
STM4149	rplK	1.8590077	1.12E-05	50S ribosomal subunit protein L11
STM2827	alas	1.8586661	2.72E-06	alanyl-tRNA synthetase
STM1320	ydjN	1.8563672	1.03E-05	putative sodium:dicarboxylate symporter
STM2431	ptsH	1.854824	3.14E-08	PTS family, Hpr protein, phosphohistidinoprotein-hexose phosphotransferase
STM4028	yihZ	1.8517909	6.73E-06	D-Tyr-tRNA(Tyr) deacylase
STM1045		1.8502955	0.061189	Gifsy-2 prophage; probable minor tail protein
STM3104	yggW	1.8409748	2.48E-06	putative oxidase
STM3656	glyQ	1.8409725	1.42E-05	glycine tRNA synthetase, alpha subunit
STM3384	yhdG	1.8407779	3.05E-05	predicted TIM-barrel enzyme
PSLT046		1.8403426	2.44E-04	putative carbonic anhydrase

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
PSLT093	ygeA	1.8388822	4.94E-06	contains similarity to *E. coli* plasmid R100 protein (YGEA) (GB:BAA78868.1)
STM1070	ompA	1.838583	4.83E-06	putative hydrogenase
STM1267		1.8302698	1.03E-05	putative cytoplasmic protein
STM3869	atpF	1.8285747	1.03E-04	membrane-bound ATP synthase, F0 sector, subunit b
STM4360	miaA	1.8266888	8.36E-06	delta(2)-isopentenylpyrophosphate tRNA-adenosine transferase
PSLT066	ssb	1.8260834	9.46E-06	single-strand DNA-binding protein
STM3742	spot	1.8238935	1.23E-10	bifunctional : (p)ppGpp synthetase II
STM4092	hslV	1.8133116	4.27E-06	peptidase component of the HslUV protease
STM1231	phoP	1.8124366	9.44E-19	response regulator in two component regulatory system with PhoQ
STM4379	yjfO	1.8114326	4.02E-06	putative lipoprotein
STM4086	glpK	1.8101674	0.001434	glycerol kinase (2nd module)
STM3570	ftsE	1.8063606	5.81E-05	putative ABC superfamily (ATP binding) transport protein
STM1197	fabF	1.8016672	5.99E-06	3-oxoacyl-[acyl-carrier-protein] synthase II
STM2378	fabB	1.7975746	6.67E-06	3-oxoacyl-[acyl-carrier-protein] synthase I
STM0833	ompX	1.7967349	1.76E-05	outer membrane protease, receptor for phage OX2
STM4361	hfq	1.7925671	1.17E-07	host factor I for bacteriophage Q beta replication, a growth-related protein
STM3699	cysE	1.787349	0.007669	serine acetyltransferase (2^{nd} module)
STM4151	rplJ	1.7870678	1.13E-05	50S ribosomal subunit protein L10
STM4147	secE	1.7863325	5.30E-05	preprotein translocase IISP family, membrane subunit
STM3127		1.7862653	4.00E-06	hypothetical protein
STM3375	yhdA	1.7806756	8.50E-06	putative diguanylate cyclase
STM0154	lpdA	1.777904	1.43E-05	lipoamide dehydrogenase (NADH)
STM2430	cysK	1.7777921	1.07E-05	subunit of cysteine synthase A and O-acetylserine sulfhydrolase A
PSLT094	trbC	1.7736906	3.81E-05	72% identity with *E. coli* plasmid F periplasmic protein (TRBC) (SW:P188473)

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM1078		1.7726812	1.37E-05	hypothetical protein
STM2338	pta	1.7665468	1.09E-05	phosphotransacetylase (2nd module)
STM2446		1.7631698	7.39E-06	hypothetical protein
PSLT045	sprA	1.7602062	1.73E-04	*S.* Typhimurium virulence plasmid resolvase homolog
STM2321	nuoI	1.7601364	9.15E-06	NADH dehydrogenase I chain I
STM0229	lpxB	1.7595026	2.46E-05	tetraacyldisaccharide-1-P
STM3415	rpoA	1.7556123	1.91E-05	RNA polymerase, alpha subunit
STM3842	yidC	1.7546129	7.34E-05	hypothetical protein
STM1000	asnS	1.7479961	4.06E-05	asparagine tRNA synthetase (3rd module)
STM3703	yibN	1.7477435	5.07E-05	hypothetical protein
STM0731		1.7466435	9.33E-06	hypothetical protein
STM2283	glpT	1.7396439	6.24E-06	MFS family, sn-glycerol-3-phosphate transport protein
STM2366	accD	1.7379955	5.44E-05	acetylCoA carboxylase, beta subunit
STM3917	rho	1.7379417	1.16E-05	transcription termination factor Rho; polarity suppressor (2nd module)
STM0013	dnaJ	1.7351342	9.38E-04	heat shock protein, DnaJ and GrpE stimulates ATPase activity of DnaK
STM3871	atpB	1.7347157	3.12E-04	membrane-bound ATP synthase, F0 sector, subunit a, important for FO assembly
PSLT001		1.7287493	9.74E-06	contains similarity to *S.* Typhimurium virulence plasmid protein
STM0152	aceE	1.7257313	3.14E-05	pyruvate dehydrogenase, decarboxylase component
STM3740	gmk	1.7234132	2.71E-05	guanylate kinase
STM1814	minC	1.7220701	1.79E-05	cell division inhibitor; activated MinC inhibits FtsZ ring formation
STM0945	clpA	1.7206893	8.27E-06	ATP-binding subunit of serine protease (2nd module)
STM1085	yccA	1.7189491	8.26E-06	putative TEGT family carrier/transport protein (2nd module)
STM0973	pflB	1.718565	1.06E-05	pyruvate formate lyase I, induced anaerobically (2nd module)
STM2439	yfeL	1.715928	1.34E-05	putative membrane carboxypeptidase (penicillin-binding protein)

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM3317	yrbK	1.7143879	1.61E-05	hypothetical protein
STM3421	rplO	1.7141607	1.02E-04	50S ribosomal subunit protein L15
STM3280	dead	1.7133908	7.77E-05	cysteine sulfinate desulfinase (1st module)
STM4364	hflC	1.71032	3.65E-05	with HflK, part of modulator for protease specific for FtsH phage lambda cII repressor
STM1830	manX	1.7066202	1.01E-05	Sugar Specific PTS family, mannose-specific enzyme IIAB (2nd module)
STM3090	metK	1.7058102	5.02E-05	methionine adenosyltransferase 1 (AdoMet synthetase)
STM2451	hemF	1.702637	4.17E-05	coproporphyrinogen III oxidase
STM0981	rpsA	1.6994942	4.72E-05	30S ribosomal subunit protein S1
STM4030		1.6967691	9.76E-06	hypothetical protein
STM0418	nusB	1.6939263	4.65E-05	transcription termination; L factor
STM3440	rplC	1.6888977	0.001217	50S ribosomal subunit protein L3
STM3966		1.6826746	2.01E-04	putative arylsulfatase regulator
STM3299	greA	1.6806388	4.11E-05	transcription elongation factor, cleaves 3' nucleotide of paused mRNA
STM3872	atpI	1.6801637	8.13E-07	membrane-bound ATP synthase subunit, F1-F0-type proton-ATPase
STM4038	fdhD	1.6799928	2.78E-05	putative formate dehydrogenase formation protein
STM2363	cvpA	1.6791147	1.99E-05	membrane protein required for colicin V production
STM4570	deoD	1.6778882	2.71E-05	purine-nucleoside phosphorylase
STM3449	yheL	1.6754541	1.78E-05	hypothetical protein
STM4319	phoN	1.6735435	1.65E-05	non-specific acid phosphatase
STM0046	ileS	1.670197	2.13E-05	isoleucine tRNA synthetase
PSLT096	trbE	1.6700864	4.37E-04	similar to *E. coli* plasmid F protein (TRBE) (SW:Q05807)
PSLT111	finO	1.6689144	4.15E-05	76% identity with *E. coli* plasmid IncFII ColB2 fertility inhibition protein (FINO) (SW:Q05781)
STM3973	tatA	1.6652113	3.47E-05	component of Sec-independent protein secretion pathway
STM3041	prfB	1.6620988	4.88E-05	peptide chain release factor RF-2 (2^{nd} module)

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM1335	rpmI	1.6582074	5.25E-05	50S ribosomal subunit protein L35
STM0089	apaG	1.6574502	3.73E-05	hypothetical protein
STM2880		1.6522231	3.07E-05	hypothetical protein
PSLT068		1.6509376	2.48E-05	*E. coli* plasmid R100 protein (YDEB) (TR:Q9WTF6)
STM0378	yaiY	1.6509327	2.02E-05	hypothetical protein
STM1325	ydiZ	1.6498965	0.175084	hypothetical protein
STM2523	gcpE	1.6495566	4.58E-05	hypothetical protein
STM2409	nupC	1.648988	1.72E-05	NUP family, nucleoside transport
STM1222	potD	1.6476932	2.08E-05	ABC superfamily, spermidine/putrescine transporter
STM2581	rnc	1.6464149	1.27E-04	RNase III, ds RNA
STM0504	ybbN	1.6456453	1.51E-05	paral putative thioredoxin protein
PSLT107		1.6449705	2.48E-05	93% identity with *E. coli* plasmid F
STM4148	nusG	1.6418349	3.91E-05	component in transcription antitermination
STM3287	nusA	1.6393031	4.30E-05	transcription pausing; L factor
STM0740	cydA	1.6368748	4.14E-05	cytochrome d terminal oxidase, polypeptide subunit I
STM3414	rplQ	1.6351483	1.81E-04	50S ribosomal subunit protein L17
STM3315	yrbH	1.6329587	0.043393	putative polysialic acid capsule expression protein
PSLT070	psiA	1.631886	1.86E-05	contains similarity to *E. coli* plasmid R6-5 protein (PSIA) (SW:P17976)
STM3108	yggL	1.6309294	7.28E-05	hypothetical protein
STM2239		1.6307073	1.93E-05	putative phage protein; homology to antiterminator protein Q of phage P5
STM4557	hold	1.6302068	2.75E-05	DNA polymerase III, psi subunit
STM4008		1.6294292	9.26E-05	hypothetical protein
STM0669	phoL	1.6294109	2.35E-05	putative phosphate starvation-inducible protein, ATP-binding (2nd module)
STM4393	rpsR	1.62727	2.44E-04	30S ribosomal subunit protein S18

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM2367	dedA	1.6262411	6.23E-05	hypothetical protein
STM3898	yifE	1.626067	3.25E-05	putative LysR type transcriptional regulator with PssR
STM0407	secD	1.6225219	3.42E-05	preprotein translocase, IISP family, part of the channel
STM1831	many	1.6220734	3.67E-05	Sugar Specific PTS family, mannose-specific enzyme IIC (1st module)
STM0104	araC	1.619043	7.50E-05	transcriptional regulator (AraC/XylS family) for ara operon (2nd module)
STM1777	hemA	1.6162349	4.29E-05	glutamyl tRNA reductase
PSLT097	traF	1.6126336	7.76E-05	86% identity to aa 10-247 of *E. coli* plasmid F pilus assembly protein (TRAF) (SW:P14497)
PSLT069	psiB	1.6097664	1.43E-04	53% identity to *E. coli* plasmid R100 protein (PSIB) (GB:X12463)
STM3997	dsbA	1.6094499	9.91E-05	periplasmic protein disulfide isomerase I
STM3015	ygeA	1.6061969	4.39E-05	putative aspartate racemase
STM3710	rfaD	1.6061337	4.48E-05	ADP-L-glycero-D-mannoheptose-6-epimerase
PSLT109		1.6049771	7.14E-05	Pseudogene:
STM0559	rfbI	1.604317	4.43E-05	putative glycosyl translocase
STM4346	yjeO	1.6024747	5.90E-05	hypothetical protein
STM3433	rplP	1.601039	1.72E-04	50S ribosomal subunit protein L16
STM3040	lysS	1.5996742	4.76E-05	lysine tRNA synthetase, constitutive (2nd module)
PSLT005	tap	1.5985746	2.09E-04	*S.* Typhimurium virulence plasmid protein (TAP) (GB:AAB53038.1)
STM0188	ligT	1.5984762	2.79E-05	2'-5' RNA ligase
STM4087	glpF	1.5978675	1.50E-04	MIP channel, glycerol diffusion
STM3311	yrbD	1.5949146	8.06E-05	putative ABC superfamily transport protein
STM1186		1.594236	4.55E-05	pseudogene; in-frame stop following codon 97; no start near coli start
STM0473	hha	1.5924951	2.57E-05	hemolysin expression modulating protein (involved in environmental regulation of virulence factors)
STM2952	eno	1.5922781	0.002338	enolase
STM3089	yqgD	1.5919503	2.64E-05	hypothetical protein

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM3286	infB	1.5903343	6.31E-05	protein chain initiation factor IF-2 (2nd module)
STM0735	sdhB	1.5893592	2.97E-05	succinate dehydrogenase , Fe-S protein
STM3441	rpsJ	1.5891519	1.06E-04	30S ribosomal subunit protein S10
STM2323	nuoG	1.5867194	3.90E-04	NADH dehydrogenase I chain G
STM3282	pnp	1.5864259	2.33E-04	polynucleotide phosphorylase, member of mRNA degradosome
STM4127	yijC	1.586153	6.06E-05	putative negative regulator
STM1925	flhD	1.5860658	6.89E-05	regulator of flagellar biosynthesis, acts on class 2 operons
STM3372	mreD	1.5858037	3.64E-05	rod shape determining protein
STM3655	glyS	1.5846469	5.79E-04	glycine tRNA synthetase, beta subunit (3rd module)
PSLT105	trbH	1.5828831	1.41E-04	72% identity with *E. coli* plasmid F protein (TRAH) (SW:P19381)
STM4276		1.5814494	6.45E-05	hypothetical protein
STM2809	proV	1.5808909	2.91E-05	ABC superfamily, glycine/betaine/proline transport protein (1st module)
STM0628	pagP	1.5805324	4.12E-05	PhoPQ-activated gene
PSLT110	traX	1.5769912	1.04E-04	72% identity to aa 7-248 of *E. coli* plasmid IncFII R100 and R6-5 protein (TRAX) (SW:P22710)
STM1349	pps	1.5737809	4.59E-05	phosphoenolpyruvate synthase (3rd module)
STM2953	pyrG	1.5736188	1.80E-04	CTP synthetase
STM0542	fold	1.5715878	4.75E-05	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase
STM3380	accC	1.5711156	1.27E-04	acetyl CoA carboxylase, biotin carboxylase subunit (2nd module)
STM0158	acnB	1.5677694	1.51E-04	aconitate hydratase 2 (2nd module)
STM2081	gnd	1.5666664	1.27E-04	gluconate-6-phosphate dehydrogenase, decarboxylating (1^{st} module)
STM2585		1.5657952	1.50E-04	Gifsy-1 prophage: similar to transpose
STM3526	glpD	1.5652484	1.37E-04	sn-glycerol-3-phosphate dehydrogenase (aerobic)
STM2925	nlpD	1.5640013	3.81E-05	lipoprotein (2nd module)
STM0667	ybeX	1.5596569	4.52E-05	putative integral membrane protein

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM0166	speE	1.5591496	0.006848	spermidine synthase (putrescine aminopropyltransferase)
STM2214	spr	1.5573742	4.97E-05	putative lipoprotein
STM3438	rplW	1.555051	3.05E-04	50S ribosomal subunit protein L23
STM3369	yhdP	1.554503	1.39E-04	paral putative protease (3rd module)
STM4561	osmY	1.5542585	6.88E-05	hyperosmotically inducible periplasmic protein, RpoS-dependent stationary phase gene
STM2686	yfjF	1.5511385	1.08E-04	hypothetical protein
STM2944	ygcB	1.5503219	0.032886	hypothetical protein; predicted helicase
STM3184	yqiB	1.5493659	4.89E-05	*E. coli* unique protein
STM4366	purA	1.5490919	9.60E-05	adenylosuccinate synthetase
STM3925	wecE	1.5486572	5.87E-05	TDP-4-oxo-6-deoxy-D-glucose transaminase (1st module)
STM0474	ybaJ	1.5482496	9.23E-05	orf
PSLT052	parA	1.5475043	7.36E-04	69% identity with *E. coli* plasmid P1 partitioning protein A (PARA) (SW:P07620)
STM0485	ybaB	1.5456855	5.67E-05	hypothetical protein
STM1345	ydiU	1.5447199	8.81E-05	hypothetical protein
STM3978	yigC	1.543027	1.19E-04	putative oxidoreductase
STM2282	glpQ	1.5405587	5.99E-05	glycerophosphodiester phosphodiesterase, periplasmic
STM0469		1.5391681	0.063133	putative 50S ribosomal protein L31 (second copy)
STM2971	sdaB	1.5383602	2.74E-04	L-serine dehydratase (L-threonine deaminase 2) (2nd module)
STM3731	dut	1.5370923	1.07E-04	deoxyuridinetriphosphatase
STM1190	yceD	1.533149	9.82E-05	predicted metal-binding
STM0698	pgm	1.5313249	9.35E-05	phosphoglucomutase (1^{st} module)
STM1159	yceO	1.5306616	1.90E-04	hypothetical protein
STM0211	yaeH	1.5303458	7.25E-05	hypothetical protein
STM2319	nuoK	1.5291668	2.26E-04	NADH dehydrogenase I chain K

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM0171	yadF	1.5287195	2.34E-04	putative carbonic anhydrase
STM2362	purF	1.528376	6.45E-05	amidophosphoribosyltransferase (PRPP amidotransferase) (1st module)
STM2924	rpoS	1.5282947	3.70E-09	sigma S (sigma 38) factor of RNA polymerase, major sigma actor during stationary phase
STM3122		1.5280744	8.49E-05	putative arylsulfatase
STM3285	rbfA	1.5280362	9.69E-05	ribosome-binding factor, role in processing of 10S rRNA
STM0941	ybjY	1.5262523	1.63E-04	paral putative membrane protein
STM3862	glum	1.5255368	1.85E-04	N-acetyl glucosamine-1-phosphate uridyltransferase
STM3445	tufA	1.5252715	4.58E-04	protein chain elongation factor EF-Tu (duplicate of tufB) (1st module)
STM0615	ybdR	1.5251933	9.45E-05	putative dehydrogenase
STM3849	yieE	1.5244334	6.46E-05	hypothetical protein
STM2519	engA	1.5233915	7.47E-05	putative GTP-binding protein
STM3915	trxA	1.520948	2.52E-04	thioredoxin 1, redox factor
STM1196	acpP	1.5199347	1.56E-04	acyl carrier protein
PSLT071		1.519874	6.69E-05	Pseudogene
STM0990	ycbC	1.5198201	7.43E-05	putative KicA protein
PSLT099	trbB	1.5178262	2.85E-04	78% identity with *E. coli* plasmid F protein (TRBB) (SW:P18035)
STM2661	yfiH	1.5164467	2.98E-04	hypothetical protein
STM4129	trmA	1.5154892	1.41E-04	tRNA (uracil-5-)-methyltransferase(2nd module)
STM0136	secA	1.5127856	0.001198	preprotein translocase; secretion protein of IISP family
STM2093	rfbI	1.5109097	8.94E-05	LPS side chain defect: CDP-6-deoxy-delta3,4-glucoseen reductase
STM2630		1.5082079	2.38E-04	Gifsy-1 prophage
STM1907	cutC	1.5077773	8.48E-05	copper homeostasis protein
STM0230	rnhB	1.5072541	8.03E-05	RNAse HII
STM0668	ybeY	1.5055393	1.34E-04	orf

Systematic Name	Common Name	Normalised Ratios	t-test P-value	Description
STM4568	deoA	1.5053499	9.68E-05	thymidine phosphorylase
STM3920	wecB	1.5050204	2.40E-04	UDP-N-acetyl glucosamine -2-epimerase
STM1338	pheT	1.5038403	8.09E-05	phenylalanine tRNA synthetase, beta-subunit
STM1337	pheS	1.5019591	1.83E-04	phenylalanine tRNA synthetase, alpha-subunit
STM3702	grxC	1.5000141	4.93E-04	glutaredoxin 3

Table 6 – A list of all genes from the IFR microarrays with a normalised ratio greater than 1.5 ($p<0.05$). The remainder of this dataset can be found in the appendix of this thesis.

Gene Name	Fold Induction	Predicted Function
clpX	8.756818771	ATP-dependent specificity component of clpP serine protease
surA	5.918399811	survival protein
yaeT	5.782641411	hypothetical protein
rpoD	5.156614304	major sigma factor during exponential growth
ftsZ	4.740665913	forms circumferential ring in cell division
STM1253	4.250430584	Putative cytochrome
ybhQ	4.24796772	hypothetical protein
plsB	4.007797718	Glycerol-3-phosphate acyltransferase
dnaK	3.346360207	Heat shock protein
mopA	3.171142578	chaperone Hsp60 with peptide-dependent ATPase activity
secY	3.12131691	preprotein translocase of IISP family
rpsG	3.090647697	30S ribosomal subunit protein S7
fusA	3.034047365	protein chain elongation factor EF-G
rplE	3.015765667	50S ribosomal subunit protein L5
rpsK	2.943320274	similar to ribosomal subunit protein S11
rplB	2.871021986	50S ribosomal subunit protein L2
rplF	2.611186504	50S ribosomal subunit protein L6
rimM	2.534205914	16S rRNA processing protein
ftsA	2.470084667	ATP-binding cell division protein
hflB	2.431609631	ATP-dependent zinc-metallo protease
lpxA	2.265328646	UDP-N-acetylglucosamine acetyltransferase
cca	2.194531918	tRNA nucleotidyl transferase
clpB	2.165037394	ATP-dependent protease
hflK	2.125346422	FtsH phage lambda cII repressor
yfgE	2.10953784	ATPase involved in DNA replication initiation
rplD	2.070434809	50S ribosomal subunit protein L4
rpmD	2.02807188	50S ribosomal subunit protein L30
rpmJ	1.987841368	50S ribosomal subunit protein X
trmD	1.938712239	tRNA (guanine-7-)-methyltransferase
fabG	1.923911214	3-oxoacyl-[acyl-carrier-protein] reductase
cyoD	1.907806277	cytochrome o ubiquinol oxidase subunit IV
tsf	1.88843751	protein chain elongation factor EF-Ts
rpsE	1.881262422	30S ribosomal subunit protein S5
alas	1.858665943	alanyl-tRNA synthetase
glyQ	1.840972662	glycine tRNA synthetase, alpha subunit
fabB	1.797574759	3-oxoacyl-[acyl-carrier-protein] synthase I
lpdA	1.777904034	lipoamide dehydrogenase (NADH)
rpoA	1.755612254	RNA polymerase, alpha subunit
yidC	1.754613042	putative preprotein translocase subunit YidC
asnS	1.747995973	asparagine tRNA synthetase
rplO	1.714160681	50S ribosomal subunit protein L15
metK	1.70581007	methionine adenosyltransferase 1
rplC	1.688897848	50S ribosomal subunit protein L3
ileS	1.67019701	isoleucine tRNA synthetase

Gene Name	Fold Induction	Predicted Function
prfB	1.662098765	peptide chain release factor RF-2
yaiY	1.65093267	hypothetical protein
nusA	1.639303088	L factor
rplP	1.601039052	50S ribosomal subunit protein L16
lysS	1.599674225	constitutive lysine tRNA synthetase
eno	1.592278123	enolase
infB	1.590334296	protein chain initiation factor IF-2
glyS	1.58464694	glycine tRNA synthetase, beta subunit
pyrG	1.57361877	CTP synthetase
fold	1.57158792	5,10-methylene-tetrahydrofolate cyclohydrolase
ybeX	1.559656858	putative CBS domain-containing protein
yigC	1.543027043	putative oxidoreductase
ybdR	1.525193334	putative dehydrogenase
engA	1.523391485	putative GTP-binding protein
trxA	1.520948052	thioredoxin 1
pheT	1.503840327	phenylalanine tRNA synthetase, beta-subunit

Table 7 - A list of essential genes in *S.* Typhimurium significantly upregulated after σ^E overexpression as described by (Knuth *et al.*, 2004).

Figure 16 illustrates the effect σ^E overexpression has on other periplasmic and cytoplasmic stress response regulators. As expected *rpoE* is the most upregulated due to overexpression in our experimental design. Neither of the other periplasmic responses described thus far, *cpxRA* and *baeSR* are affected at the transcriptional level by overexpression of σ^E. *rpoH* and *rpoD* are both significantly upregulated greater than 3 fold, and this is in agreement with characterisation of the *E. coli* σ^E regulon as well as the other data presented in this study. Interestingly *rpoS* and *rpoN* are also both significantly upregulated but only at the cut off of 1.5 fold. As you will see later in this chapter neither of these genes possess *rpoE*-like promoters in their regulatory regions, so this effect is probably indirect.

When categorised in accordance with the Monica Riley gene function classification system (Riley, 1993), 30% of the genes significantly upregulated greater than 1.5 fold have a function associated with the cell envelope (Figure 17).

5.4 Promoter Consensus Search

Based upon the *rpoEP3* promoter and from known σ^E dependent promoters of other genes such as *htrA* and *rpoH* we constructed a promoter consensus (figure 17B) which could be used to screen the *S.* Typhimurium genome for other putative σ^E regulated promoters, using degenerative nucleotide strings in Artemis (Rutherford *et al.*, 2000).The consensus used contained a 16bp spacer between the -35 and -10 binding regions, as reduction of this spacer to 15bp has been shown to reduce the activity of the *rpoEP3* promoter (Miticka *et al.*, 2004). We also used the *S.* Typhimurium *rpoEP3* promoter as a template for a mismatched pattern search (Chaudhuri, Khan, and Pallen, 2004). Table 8 lists the genes that were identified with this promoter search, along with

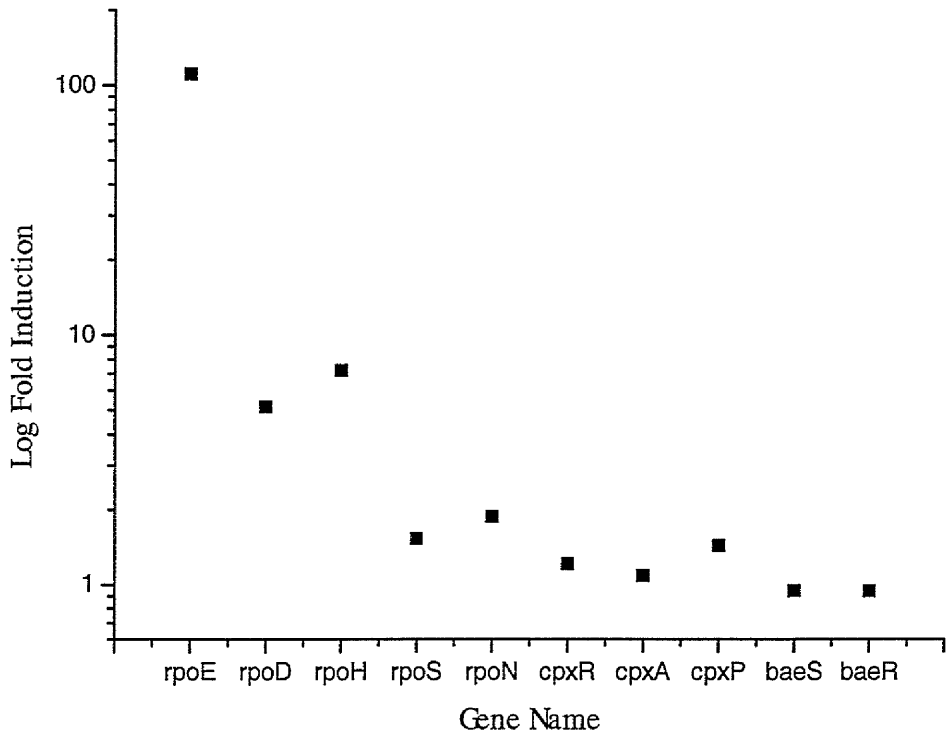


Figure 16. Graph showing the log fold induction determined by array analysis after overexpression of σ^E, of genes implicated in a variety of *S.* Typhimurium stress responses.

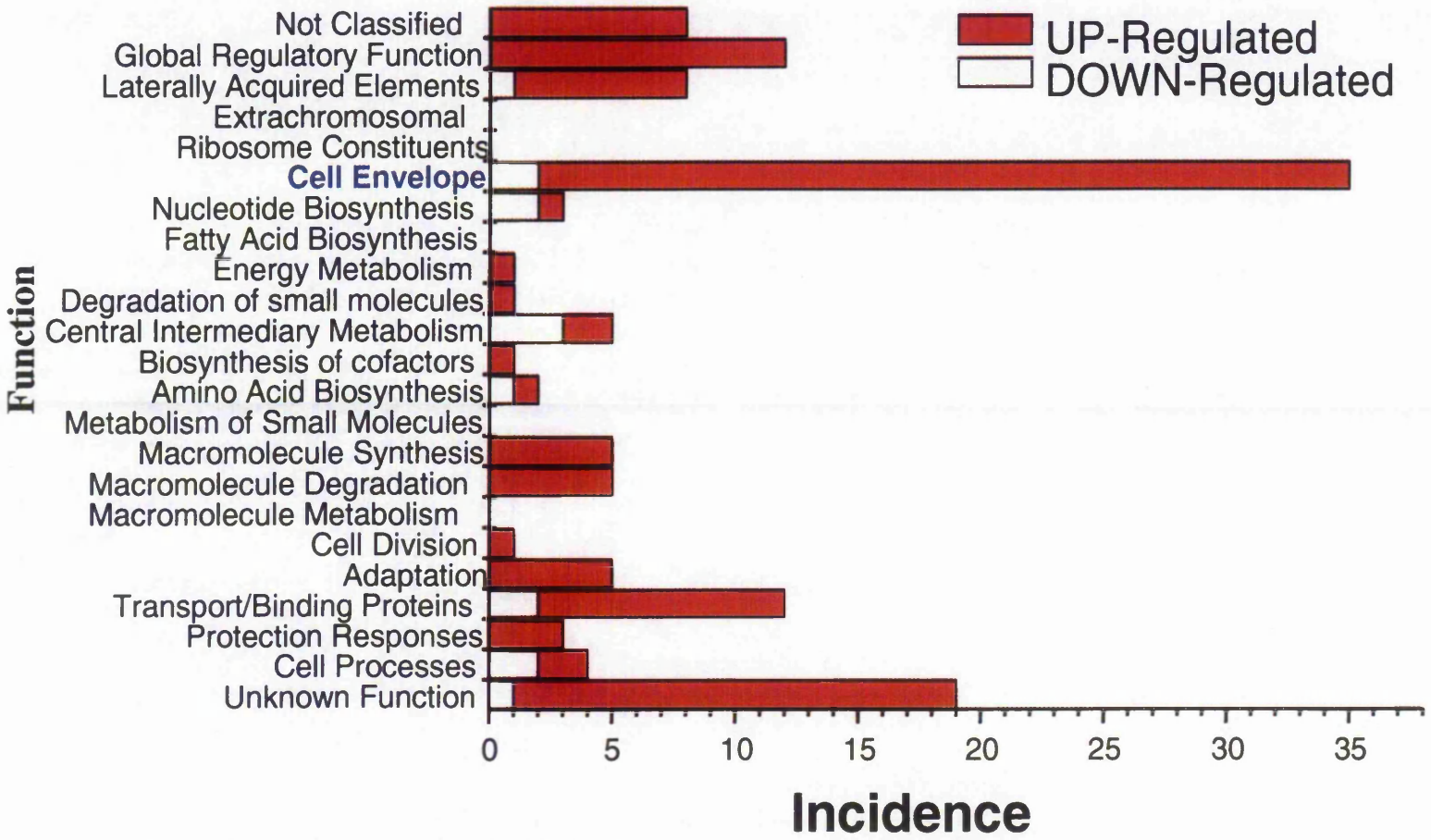


Figure 17. Graph depicting the functional classification of genes significantly up and down regulated after overexpression of σ^E. Functional groups are based on the Monica Riley classification system (Riley, 1993)

Figure 17B. The promoter sequence used in our promoter consensus search.

This sequence was derived from a number of putatively RpoE regulated genes. The degenerative code is based upon IUPAC code (Chaudhuri, Khan, and Pallen, 2004) where B=G or T or C, V=G or C or A, Y=T or C, W=A or T, N=G or A or T or C. 16N relates to a 16bp spacer between the -35 and -10 regions. Highlighted nucleotides appear to be highly conserved (Miticka *et al.*, 2004).

Gene Name	Predicted Function	putative promoter -35 N(16) -10	Promoter Location	σ^E Array Fold Induction	Two Plasmid[1]	Identified in *E. coli*	PB Array	Macrophage Array
rpoE	Alternative Sigma factor	GGAACTT TCTAA	-110	111.6	Y	Ya		up
htrA	Periplasmic serine protease	GGAACTT TCTGA	-73	32.8	Y	Ya	up	up
yfiO	Lipoprotein	GGAACAT TCTAA	-216	7.87	Y	Ya		down
rpoH	Alternative sigma factor	TGAACTT TCTGA	-120	7.2	Y	Ya		up
yggN	Periplasmic protein	CGAACTT ACTAA	-77	9.5		Ya	up	up
tm2447	Outer membrane lipoprotein	GGCACTT TCCAA	-60	12.11			up	up
dedD	Lipoprotein	GGAACAT TCTAT	-152	7.2	Y			
slp	Outer membrane protein	GAAACTT TCTCA	-93	6.10				up
yggT	Integral membrane protein	GGCACTT TCCAA	-488	3.68	Y			down
lpdA	Lipoamide dehydrogenase	GGAACTA TGTTA	-424	1.77				down
tolR	Outer membrane integrity	GGAACAA TCAAA	-91	4.3	Y			
ycbK	Outer membrane protein	AGAAGTT CCTAA	-255	NA	Y			
fabG	Acyl carrier protein	GGAATTT TTGAA	-160	1.92				
fabF	Acyl carrier protein	GAAATTT TCGAA	-335	1.80				up
tm1251	Molecular chaperone	GAAACAT TCTTA	-434	14.3	Y		down	up
ydiV	Diguanylate cyclase	TGAACTT TCATA	-264	4.64			up	up
ddg	Palmitoleoyl transferase	GGAACCA CCTAA	-186	5.80		Yb		up
yfeK	Periplasmic protein	GCAACTT TCTGA	-45	2.11	Y			up
tm2494	Inner membrane protein	GGAACGA TCAAA	-58	2.99			up	
cadB	Lysine transport protein	GCAACAT TGTAA	-259	3.31				up
smpA	Small membrane protein A	TAAACTT TCTGA	-151	7.70		Yb		
proV	ABC superfamily	GCAAATT TCTGA	-108	1.58				up
rpoD	Sigma D	TGAACCG TCGAA	-293	5.16	Y	Ya		
yiaD	Outer membrane lipoprotein	GGAATTT TCATA	-80	19.42			up	up
yijD	Inner membrane protein	GGAACTT TCGAA	-250	2.81				
rplA	50S ribosomal subunit	GGAATTT TACAA	-442	2.55				down
tm4276	Cytoplasmic protein	GGAACTG TCTAT	-82	1.58				up
hfq	Host factor I for bacteriophage Q	GCAATTT TCAAA	-56	1.79				
creB	Response regulator (creBC)	GGAAGCT TCAAA	-361	2.38				up

Gene Name	Predicted Function	putative promoter -35 N(16) -10	Promoter Location	σ^E Array Fold Induction	Two Plasmid[1]	Identified in *E. coli*	PB Array	Macrophage Array
surA	Ppiase	CGAACAT TCTAT	-85	5.92	Y	Ya		down
dsbG	Disulfide isomerase	TGAACAC TCTAA	-321	2.29				down
yceP	Cytoplasmic protein	ACAACTT TCAAA	-393	2.5				up
tm1254	Outer membrane lipoprotein	GGACATT TCTAT	-50	NA			up	NA
oppC	Oligopeptide transport	CGAACTT GCAAA	-328	3.09				
fliC	Flagellar biosynthesis	GGAAGTG TCGAA	-113	0.48			down	
sixA	Phosphohistidine phosphatase	GAAACTG TCAAA	-229	4.25		Yb		up
nlpB	Lipoprotein 34	GGAACTT ATCAA	-402	7.91		Ya		
yfgM	Inner membrane protein	GGAATTT TCGAT	-326	4.64				
hflB	Zinc metalloprotease	TGAACTT TGGAA	-425	2.43				
ftsJ	23s RNA methyltransferase	TGAACTT TGGAA	-274	2.11				up
greA	Transcription elongation factor	GGAACTC TCAAA	-171	1.68				up
fusA	Protein chain elongation factor	CGAACTT ACAAA	-202	3.03	Y	Yb		
fkpA	Ppiase	GAAACTA TCTGA	-139	NA	Y	Ya		NA
cpxR	Response regulator (cpxRA)	GTAACTT CCAAA	-164	1.2				
hslU	Protease	GGAAATG TCAAA	-346	1.88			down	
atpG	ATP synthase	CGAACTG TCGAA	-216	2.66				down
phoN	Acid phosphatase	TGAACTT AATAA	-447	1.67			up	up
psd	Phosphatidylserine decarboxylase	GGAACAA TCGAA	-305	5.10	Y	Yb		
yjfO	Lipoprotein	GGAACAT TCAAT	-41	1.81	Y			
bacA	Bacitracin Resistance	TAAACCA TCTTA	-71	2.02		Yb		
plsB	Glycerolphosphate acyltransferase	AGAACCT TCAAT	-76	4.02	Y			
sbmA	ABC superfamily transporter	CGAACTA TCACA	-124	3.94	Y	Yb		
ydcG	Periplasmic glucans biosynthesis	ATGATTT TCAGA	-101	2.00	Y			
yraP	Periplasmic protein	TGCACTA TCTTA	-370	18.3	Y	Ya	up	down
ygiM	Membrane protein	CGAACTT TCAGT	-197	7.18	Y	Ya	up	
tm1250	Molecular chaperone	GAAACTA TCTTT	-402	11.34	Y		up	down
ycbL	Metallo-beta-lactamase	CCAACTT TGTTA	-307	2.85				
ptr	Protease II	GCAACTT TCTAT	-616	2.05	Y			
yabI	Membrane associated	TGAATGA TCATA	-114	3.69	Y			up

Gene Name	Predicted Function	putative promoter -35 N(16) -10	Promoter Location	σ^E Array Fold Induction	Two Plasmid[1]	Identified in *E. coli*	PB Array	Macrophage Array
eno	Enolase	TGAACTG TCTGA	-924	1.59	Y			
yaeT	Inner Membrane Protein	GGAACTT TCGAA	-938	3.59		Ya		down
pqiA	Paraquat inducible	TGAACCA TCGAA	-528	1.94			up	
ttk	Transcriptional regulator	GCAACCT TCCGA	-319	2.59				down

Table 8. A list of all the genes for which a putatively σ^E regulated promoter could be identified. The -35 and -10 regions of each promoter are listed and all are separated by a 16 bp spacer. The location of the promoter is given with respect to the predicted start codon of the given gene. The normalised fold induction after overexpression of σ^E is also given. **1** refers to the two plasmid screen in *S.* Typhimurium, **a** to the screen for *rpoE* regulated promoters in *E. coli* by (Dartigalongue, Missiakas, and Raina, 2001) and **b** to the two plasmid screen in *E. coli* (Rezuchova *et al.*, 2003). PB array refers to the *S.* Typhimurium array analysis of the effects of polymixin B on gene expression (Bader *et al.*, 2003) and the macrophage array refers to array analysis of *S.* Typhimurium gene expression within J774 macrophages (Eriksson *et al.*, 2003).

the location for the promoter in relation to the first base of the corresponding genes predicted start codon, normalised expression ratios from the array analysis and whether the gene was identified by the two plasmid system in *S.* Typhimurium, or identified as σ^E regulated in *E. coli*. We have also included in this table whether the gene was up (>1.5) or down (<0.5) regulated within the macrophage according to (Eriksson *et al.*, 2003) or by the addition of the cationic peptide polymixin B as determined by (Bader *et al.*, 2003). As you can see the majority of the promoters fit a similar pattern to that described in (Miticka *et al.*, 2004), with the -35 (ggAActt) and -10 (TctaA) regions conserved in most promoters identified, although there were a few exceptions. Of the genes previously identified in *E. coli* as σ^E regulated there are a few that lack a promoter consensus directly upstream of the gene in our screen, the most significant of which are *yaeL* and *skp*, although the gene downstream of *yaeL*, *yaeT,* does appear to have a σ^E dependent promoter. From S1 mapping performed by the Kormanec laboratory subsequent to our screen (GR, data not shown), there appears to be no σ^E dependent promoter upstream of *skp*. Lack of *skp* does generate an *rpoE* inducing environment, as shown in chapter 4 and *skp* is significantly upregulated after over-expression of σ^E according to the microarray data. It therefore appears that *skp* is σ^E regulated but at this stage we are unsure whether this regulation is direct or indirect. The σ^E dependent promoters of *E. coli* identified in (Dartigalongue, Missiakas, and Raina, 2001) do not appear to fit into any consensus, and the *skp* promoter identified has an 18bp spacer with no similarity to the -10 and -35 regions of our consensus.

Fifty nine genes were identified with a promoter consensus and a significant fold (>1.5, or <0.5) change in expression on our microarrays, of which only 19 genes have previously been identified in *E. coli* and 25 identified by two plasmid screening in *S.*

Typhimurium. Of the 59 genes identified by array analysis and with a promoter consensus search, 22 were significantly upregulated within macrophages, and 12 were upregulated by the addition of polymixin B to the growth medium.

5.5 Selection of Genes for Mutational and Phenotypic Analysis

After screening for σ^E regulated genes the next step was to select genes for further analysis. It must be stressed that not all of this data was available at the early stages of this study, so some genes such as *sbmA and bacA*, were selected solely on the basis of identification in the first round of the two plasmid screen in *S.* Typhimurium. As the initial collaboration between the Kormanec group and ourselves was to perform phenotypic analysis on genes they identified the majority of the genes identified by this process have also been mutated. Other genes were selected on the basis of a number of criteria such as up-regulation on the σ^E arrays, a function indicative of σ^E regulation such as periplasmic proteases and chaperones, a good promoter consensus and a potential role in virulence. No genes were selected that had previously been mutated according to the literature.

All of the genes selected for mutation are listed in table 9, most of which were successfully constructed by red mutagenesis. The analysis of these genes makes up the remainder of the chapters of this thesis. Some of them require chapters in their own right such as *degS*, whilst others have been grouped on the basis of phenotype, particularly if attenuated in a murine model of infection. The last results chapter consists of a group of genes for which no phenotype was discovered in the assays used in this thesis.

Gene	Mutant Constructed
degS	Y
stm1251	Y
stm1250	Y
stm1254	Y
stm1263	Y
yggN	Y
yggT	Y
yaeL	N
yaeT	N
skp	Y
stm2447	N
oppD	Y
yiaD	Y
yccV	Y
ygiM	Y
bacA	Y
sbmA	Y
tolR	Y
ptr	Y
rseC	Y
yraP	Y
yabI	Y
ybbN	Y
mlc	Y
dedD	Y
yfiO	Y
yrfH	Y
ycbK	Y
plsB	N
htrA	Y
psd	N
eno	N

Table 9. Genes listed in this table were selected for mutational and phenotypic analysis. The ability to construct a mutant by red mutagenesis has been indicated. Alternative methods for mutagenesis were not tried. Five of the six genes for which mutants were unable to be constructed were listed in (Knuth *et al.*, 2004) as essential genes for cell viability in *S.* Typhimurium.

5.6 Discussion

In order for this study to be successful, identification of σ^E regulated genes in *S.* Typhimurium was of prime importance. Although (Dartigalongue, Missiakas, and Raina, 2001) claim to have characterised the σ^E regulon of *E. coli*, by using a different approach Rezuchova *et al.*, 2003 identified 11 new σ^E dependent promoters potentially directing the expression of 15 genes. Although genes were being identified by the Kormanec group using the two plasmid system in *S.* Typhimurium, their progress was slow due to the nature of the experiments involved, which is akin to a 2 hybrid screen in yeast. If we had solely relied on this method, although we had optimised the red mutagenesis system for high throughput screening, the mutagenesis and phenotypic analysis would also have been slow and also we may have missed σ^E regulated genes that may have been outwith the capacity of this screen. To overcome these problems we successfully used microarray analysis and promoter consensus screening as well as the two plasmid screen.

What became apparent from the microarray data was the complexity of the task we faced. It is easy to focus on the σ^E regulon as a single signalling pathway without considering its interactions and effects on other pathways and regulators, whether directly or indirectly. We are aware that the overexpression method we were forced to use to try and identify σ^E regulated genes was far from ideal. We would ideally have liked to use the *rpoE* mutant, and compared the expression profile of this gene to wild type *S.* Typhimurium under a known *rpoE* inducing condition. At the time of RNA preparation the only *rpoE* inducing condition that we were particularly confident in was entry into and survival within stationary phase growth, and RNA extracted from an *rpoE* culture in stationary phase was extremely poor. We are now aware of other inducing conditions such as cold shock so ideally the microarray experiments should be

repeated with RNA from these conditions. However, although we are over-expressing σ^E and intracellular copies of σ^E are, one would predict, higher than under normal inducing *rpoE* conditions our approach to the array analysis has given us a feel for the other pathways activated by induction of σ^E. With this is mind we have to be careful about assuming direct σ^E regulation from this dataset alone, and need to consider other factors. This was the main reason for conducting a promoter consensus search as we hoped it would allow us to extract genes from the array data that were directly σ^E regulated, and of course we were more confident in genes that were also identified by the two plasmid screen. This leaves us with 59 genes from the 483 genes displaying significantly differentiated expression after overexpression of σ^E. We account for the differences in these numbers through the number of global regulators whose expression was induced such as *rpoH*, *creB*, *rpoS*, *rpoN* and *spoT* which possibly leads to induction of genes in their given regulons. However, the 59 genes we have identified as putatively directly σ^E regulated gives us a much more manageable number to work with but is also far greater than the number of σ^E regulated genes thus far identified in *E. coli*.

In order to put the array data in context it was important to try and relate this wealth of data to the phenotype of an *rpoE* mutant. The most significant phenotype of the *S.* Typhimurium *rpoE* mutant is its inability to survive within a murine host. Of the 483 genes differentially expressed due to over-expression of σ^E, 50 genes have been shown to be upregulated after macrophage infection (Eriksson *et al.*, 2003), including *rpoE* itself, although none of these 50 belong to SPI-2. Of the 59 genes that are differentially regulated and have a putative promoter identified 37% (22) genes were upregulated after macrophage infection. These genes are of particular interest to us and they may prove to be potential targets to produce an attenuated *S.* Typhimurium strain. As more

array data becomes available to the public domain we can repeatedly screen our data to look for potentially σ^E regulated genes upregulated in known σ^E inducing conditions. Such a data set are the genes differentially expressed by addition of polymixin B to the culture medium (Bader *et al.*, 2003), which the *rpoE* mutant is significantly more sensitive to when compared with the wild type strain. Twelve of the 59 genes identified were up regulated by the addition of polymixin B. All of this data and information combined begins to validate our list of putatively σ^E regulated genes.

A decision had to be made however on which genes to further analyse and we had an obligation to analyse all of the genes identified by the two plasmid system. The 32 genes which we decided upon contained all of genes from the two plasmid system, although 20 genes form part of the 59 genes differentially regulated on the arrays and with a putative σ^E dependent promoter. Other genes listed such as *rseC*, *skp*, and *degS* were selected on their involvement in regulation/induction of the *rpoE* regulon, rather than their regulation by σ^E. Future studies will use the remainder of the 59 genes for phenotypic analysis. It is hoped that analysis using assays for which the *rpoE* mutant has demonstrated significant phenotype will further confirm *rpoE* regulation. Alongside this, promoter fusions containing the putatively *rpoE* regulated promoter will also be constructed and their activities compared in the wild type parent strain and the *rpoE* mutant. It is also hoped that S1 mapping will be performed by the Kormanec group on genes that have particularly interesting phenotypes.

Chapter 6 - Effect of Inactivation of *degS* on *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo*.

6.1 Introduction

The genetic locus of DegS is depicted in figure 18. DegS is a serine protease which shares homology to HtrA and DegQ, members of the HtrA family of serine proteases with chymotrypsin-like active sites, as shown in figure 19. (Bass, Gu, and Christen, 1996;Pallen and Wren, 1997)]. *degS* was initially identified in *E. coli* due to its homology with *htrA* and is reported to be under the control of σ^{70} in this bacteria, and is therefore not heat inducible (Waller and Sauer, 1996). The importance of DegS to *E. coli* viability has been a matter of some discussion, *E. coli degS* mutant strains were reported to be viable but they exhibited small colony phenotypes (Waller and Sauer, 1996), and were phenotypically variable. This was proposed to be due to accumulation of suppressor mutations (Bass, Gu, and Christen, 1996). The general agreement is now that DegS is an essential gene in laboratory strains of *E. coli* (Alba *et al.*, 2001)], and this indispensable function is through its essential regulation of the σ^{E} regulon, itself critical for *E. coli* cell viability (De Las, Connolly, and Gross, 1997a). However, in a search for genes in a pathogenic extraintestinal strain of *E. coli* genes which are required for virulence in a murine model of infection (Redford, Roesch, and Welch, 2003), *degS* was identified as being necessary for virulence. The *degS* mutant was significantly impaired in its ability to colonise the bladder and kidney in comparison with the wild type strain. This *E. coli degS* mutant was also sensitive to heat and ethanol. The authors presumed the presence of suppressor mutations on the basis of the previous studies. DegS contains a membrane anchor, a protease domain and a PDZ domain. PDZ domains generally recognise peptide sequences at the C terminus, and the PDZ domain of DegS is no exception as it recognises YYF-COOH and YQF-COOH C terminal sequences of OMP peptides (Walsh *et al.*, 2003). The crystal structures of

HtrA2 and DegP (Krojer *et al.*, 2002;Li *et al.*, 2002)] show that under non-active conditions the PDZ domain packs against the protease domain, inhibiting substrate binding and cleavage by the protease domain. Peptide binding relieves this inhibition and activates the protease activity of DegS. Active DegS cleaves the anti-sigma factor, RseA, at a valine-serine bond about 30 residues from the end of its transmembrane domain. DegS cleavage of RseA is just the first proteolytic step required for activation of the σ^E regulated stress response (Ades *et al.*, 1999;Kanehara, Ito, and Akiyama, 2003). The next step involves a second cleavage close to the cytoplasmic side of the transmembrane segment of RseA, in a reaction that depends upon the zinc protease, YaeL (EcfE). YaeL is an orthologue of the eukaryotic site-2 protease (S2P) (Bohn, Collier, and Bouloc, 2004), the metalloprotease active sites of which reside on the cytosolic side of the membrane. It spans the plasma membrane four times (Kanehara, Akiyama, and Ito, 2001), and its depletion results in the loss of viability and cell elongation (Kanehara, Ito, and Akiyama, 2002;Drew *et al.*, 2002). The physiological role of YaeL within the cell is under debate. (Dartigalongue, Loferer, and Raina, 2001) suggest a role in the degradation of σ^H and σ^E, whilst Kanehara, Ito, and Akiyama, 2002 demonstrate that YaeL is required for the positive regulation of the σ^E pathway. The fact that YaeL itself is σ^E regulated (Dartigalongue, Missiakas, and Raina, 2001) indicates that activation of the σ^E pathway is under positive feedback control, contributing to the rapid response of cells to envelope stresses. *In vitro* the cytoplasmic domain of RseA is sufficient to bind σ^E and prevent its association with the core polymerase. It is doubtful whether the second step single cleavage of RseA by YaeL is sufficient to activate the σ^E pathway. Other proteases may be required to effectively eliminate the σ^E antagonistic domain of RseA.

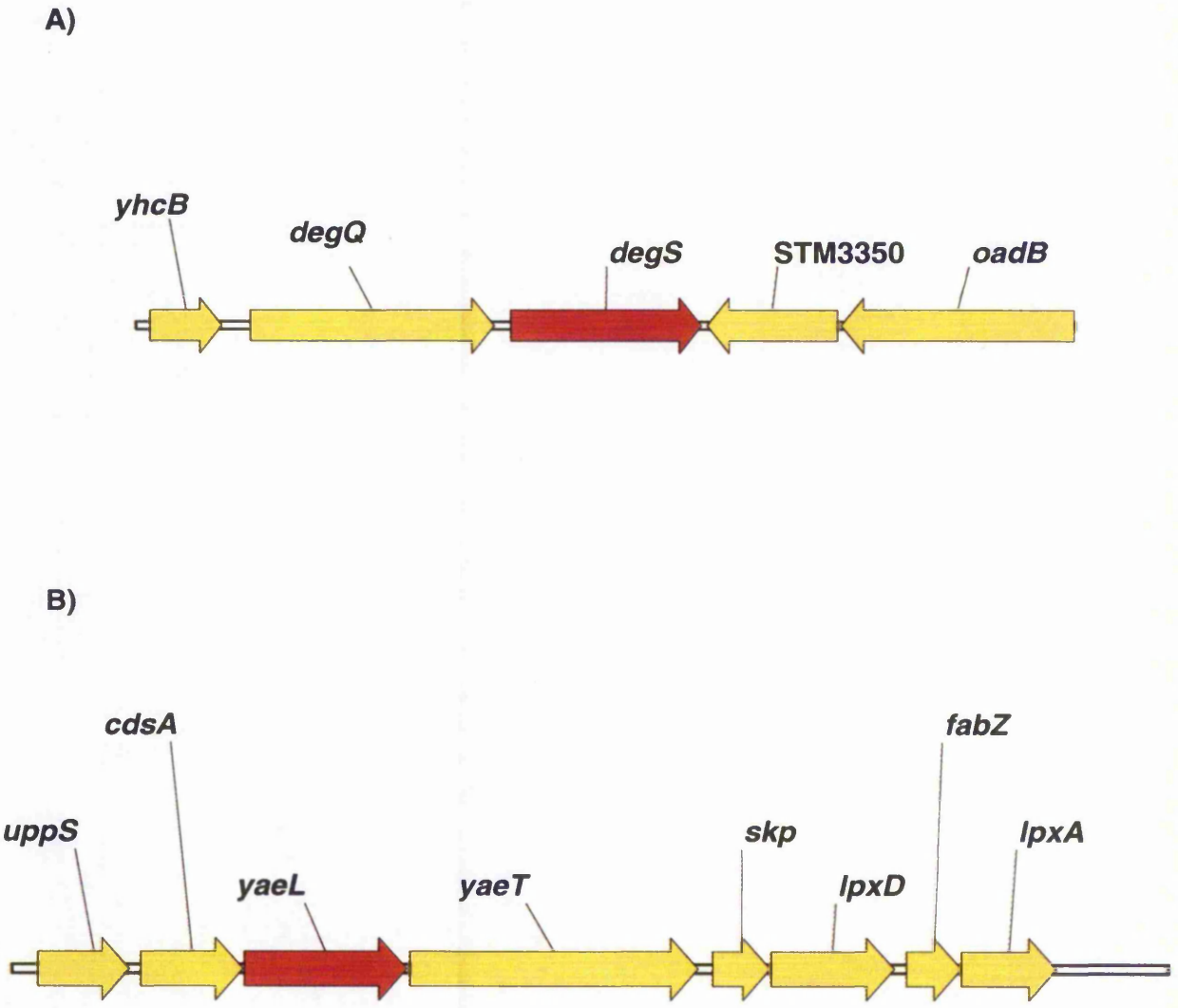


Figure 18. The genetic location of *degS* and *yaeL*

A) *degS* is located between bp 3516877-3517947 on the *S.* Typhimurium chromosome, downstream of its homologue *degQ* and upstream of the divergently transcribed *stm3350*, a putative inner membrane protein.

B) *yaeL* is located between bp 260995-262347 on the *S.* Typhimurium chromosome, downstream of *cdsA* a putative CDP-diglyceride synthase and upstream of the σ^E regulated *yaeT*, a outer membrane protein precursor.

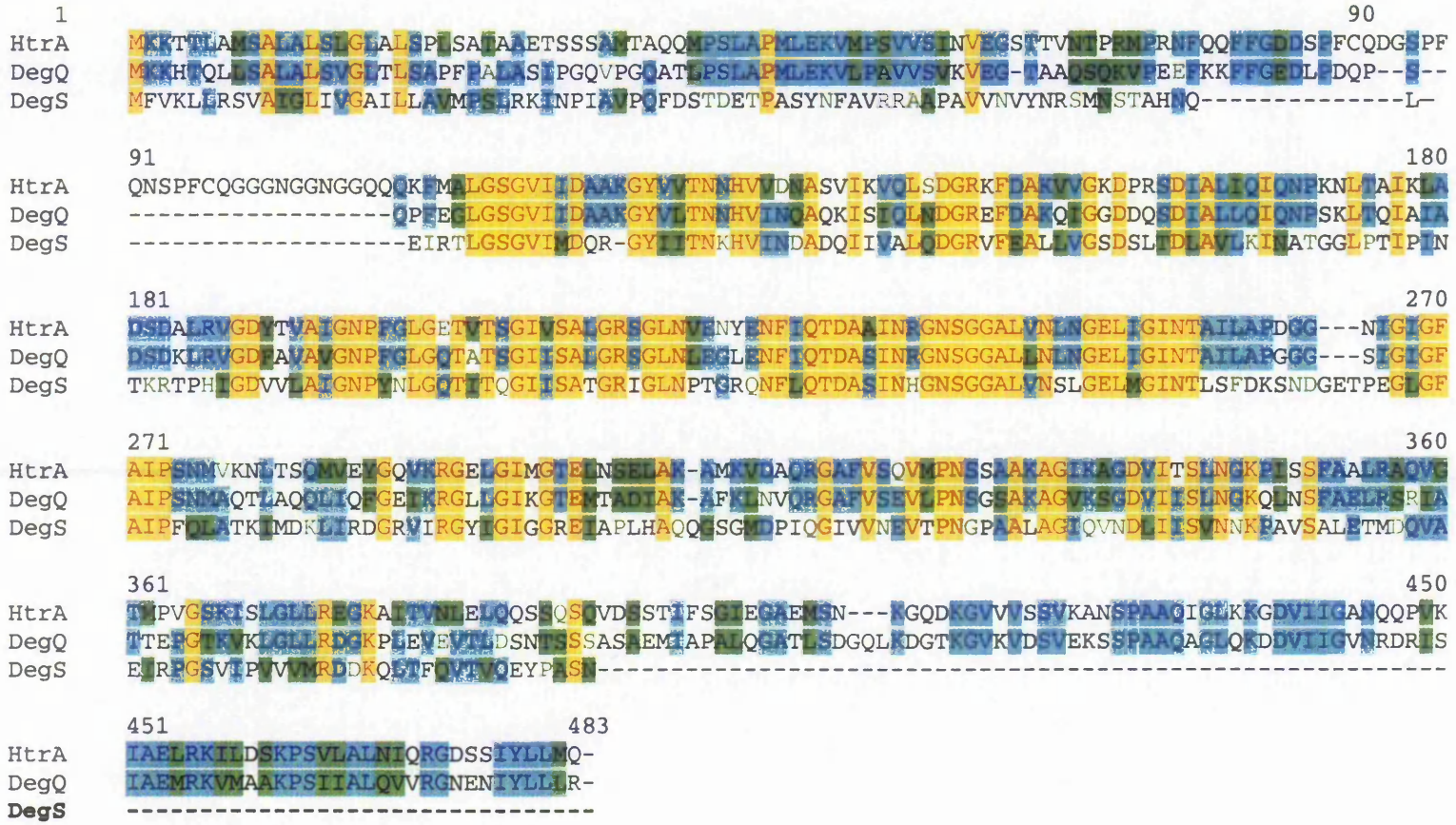


Figure 19. Sequence alignment of the amino acids of the serine protease DegS, DegQ and HtrA (DegP).
All three sequence were taken from the completed genome sequence of *S.* Typhimurium LT2a (McClelland *et al.*, 2001) and aligned using the Align X component of Vector NTI (Informax). Yellow indicates conservation in all three sequences, turquoise in two of the strains and green the presence of amino acids with similar properties although not identical.

If the sole function of these two proteins is sensing and activation of the σ^E stress response, then *degS* and *yaeL* should not be lethal mutations in *S.* Typhimurium, as survival in the absence of σ^E is feasible. In this chapter we discuss the attempted mutation of both of these genes and the characterisation of a *degS* mutant both *in vitro* and *in vivo*.

6.2 Construction of an *S.* Typhimurium *degS* Mutant

As σ^E is not essential for *S.* Typhimurium viability, we predicted that both *degS* and *yaeL* could also be successfully mutated in an otherwise wild type background, unless the gene products were necessary to perform another function unrelated to σ^E activation. Using λ Red mutagenesis we replaced the coding sequence of *S.* Typhimurium SL1344 *degS* with a kanamycin resistance cassette using oligonucleotides degSRedF and degSRedR to generate strain GVB1362. The mutation was confirmed using external oligonucleotides degSEXTF and degSEXTR as shown in figure 20. The colony morphology of the SL1344 *degS* mutant was not different from that of the wild type strain. However, after approximately 10 unsuccessful attempts at deletion of the coding sequence of *yaeL*, we predict that YaeL has another function outwith activation of σ^E in *S.* Typhimurium

6.3 Effect of the *degS* Mutation on σ^E Activity in *S.* Typhimurium.

In chapter 4 and (Miticka *et al.*, 2003) we have demonstrated that *S.* Typhimurium *rpoE* is regulated by three promoters, with the third promoter, *rpoEP3*, auto-regulated by σ^E itself. Expression of an *rpoEP3*::*lacZ* reporter gene increases in a wild type *S.* Typhimurium background during late logarithmic phase. We compared the expression

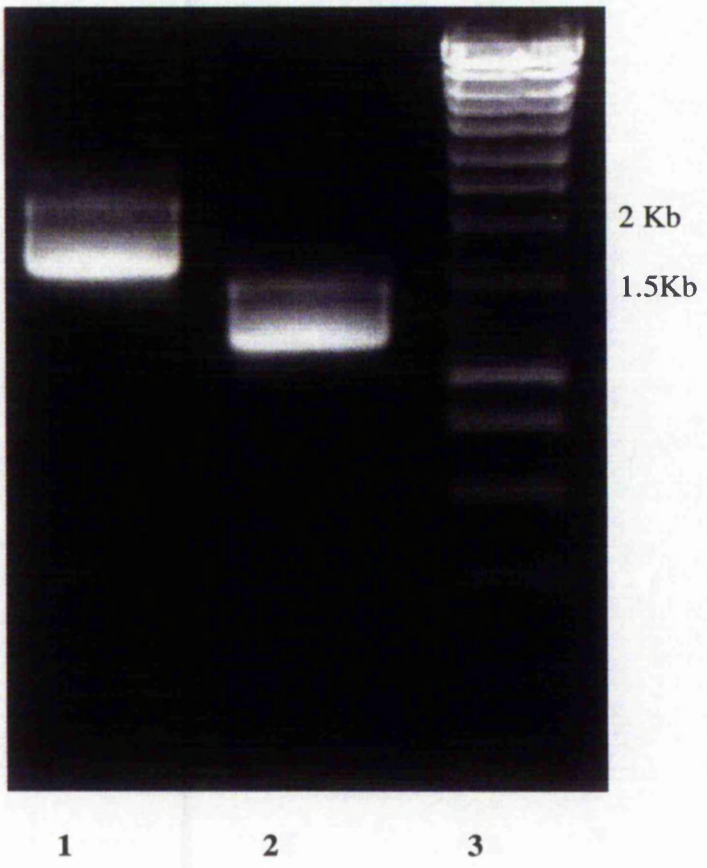


Figure 20. PCR Confirmation of a *S.* Typhimurium Δ*degS*::kan mutant .

PCR verification was performed using oligonucleotides DegSExtF and DegSExtR oligonucleotidess. The marked deletion mutant **(1)** corresponds with a 1.5Kb kanamycin cassette insert, whilst lane 2 depicts the size of the WT strain copy **(2)** of *degS* as determined by the Hyperladder I DNA ladder **(3)**.

of the *rpoEP3*::*lacZ* gene in wild type SL1344 and the isogenic *degS* and *rpoE* mutants after 6h of growth in LB broth (figure 21). Interestingly in the *degS* mutant the activity of the *rpoEP3* promoter is twice that of the *rpoE* strain ($p < 0.05$). This indicates the presence of free σ^E within the *degS* mutant, although the activity of the reporter gene is much lower than that of the wild type strain. The background β-galactosidase activity measured in the GVB311 strain is consistent of that seen with empty vector.

6.4 The *degS* Mutation Affects the Growth Rate of *S.* Typhimurium.

A *S.* Typhimurium *rpoE* mutant exhibits aberrant growth under certain conditions (Humphreys *et al.*, 1999). In particular, a *S.* Typhimurium *rpoE* mutant exhibits an extended lag phase and reaches a lower final OD when grown aerobically in LB broth. Theoretically, and on evidence derived from *E. coli degS* studies, a *degS* mutant should possess similar phenotypic characteristics as those observed previously with the *rpoE* mutant. Therefore, we compared the growth of the *degS* mutant with that of the *rpoE* mutant and the wild type strain in shaking LB-broth cultures at 37°C (Figure 22). The *degS* strain has a longer lag phase than the wild type strain although this is not as protracted as that of the *rpoE* mutant. The final OD600 of the *degS* and wild type strains were very similar. In contrast the *rpoE* mutant enters stationary phase prematurely as previously observed.

A *degS* mutant of a wild type extraintestinal isolate of *E. coli* was unable to grow in LB containing 3% ethanol (Redford, Roesch, and Welch, 2003). The wild type *E. coli* strain was able to grow under the same conditions although at a slower growth rate than in LB broth alone (Redford, Roesch, and Welch, 2003). We examined the ability of *S.* Typhimurium strains to grow in LB in the presence of 3% ethanol (figure 22). As with

E. coli, ethanol reduced the growth rate of the wild type strain relative to the growth observed in LB alone. However, unlike *E. coli*, the *S.* Typhimurium *degS* mutant was able to grow in 3% ethanol although slightly less well than the wild type strain, the difference between the strains was very similar to that seen in LB alone. The *rpoE* mutant was also able to grow in LB + 3% ethanol but its growth was more severely affected than that of the other 2 strains. The *rpoE* mutant exhibited a longer lag phase than the other strains and the exponential phase was shorter. The results show that 3% ethanol is a stressful environment for *S.* Typhimurium, but the ability of the *degS* mutant to survive and replicate in this environment is greater than that of the *rpoE* mutant.

6.5 The *degS* Mutant is Significantly More Sensitive to Hydrogen Peroxide and Polymixin B than Wild type *S.* Typhimurium.

Using the well described disc diffusion assay we attempted to discover whether a deletion of *degS* alters *S.* Typhimurium's susceptibility to cope with environments generated by oxidative stress and the antimicrobial peptide polymixin B. An *rpoE* mutant is significantly more sensitive to both of these stresses compared to the parent wild type strain (Humphreys *et al.*, 1999). Indeed, as depicted in figure 23 we discovered that the *degS* mutant is significantly, although not dramatically, more sensitive to both of these agents, than the wild type strain. (Mann Whitney Test, $p<0.001$).

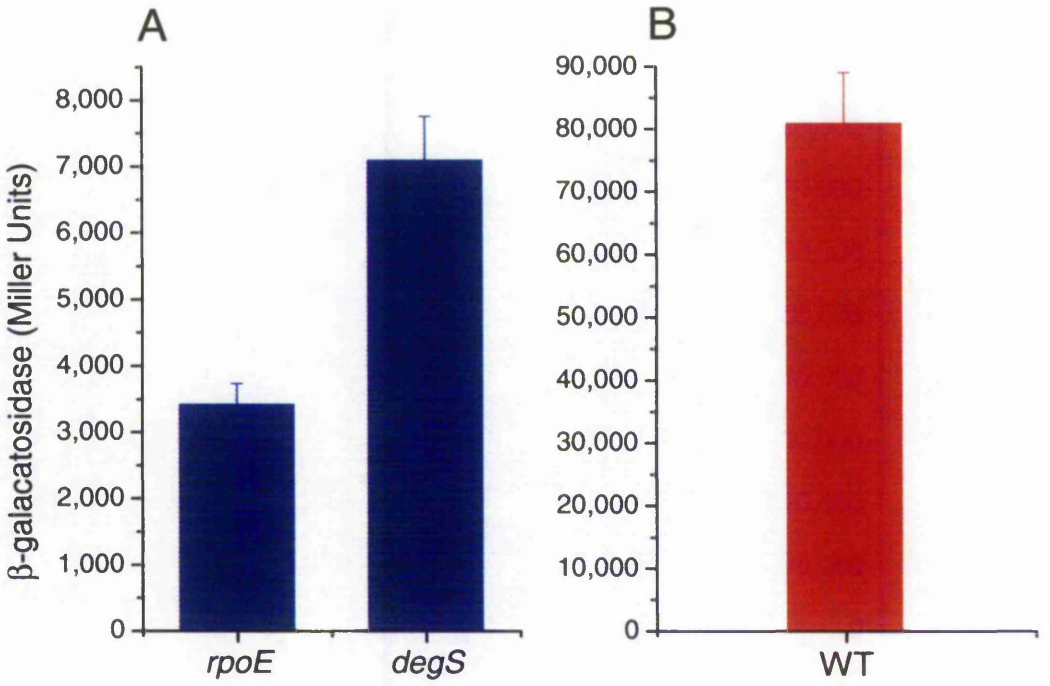


Figure 21. Effect of inactivation of *degS* on activity of the σ^E regulated pathway. β-galactosidase activities were determined for *rpoE*, *degS* (A) and WT (B) *S.* Typhimurium strains harbouring the p*rpoEP3* reporter plasmid. Strains were grown for 6h (late log phase) in LB broth at 37°C with aeration. The bars represent the mean of 7 replicates and the error bars indicate the (SD) of the mean.

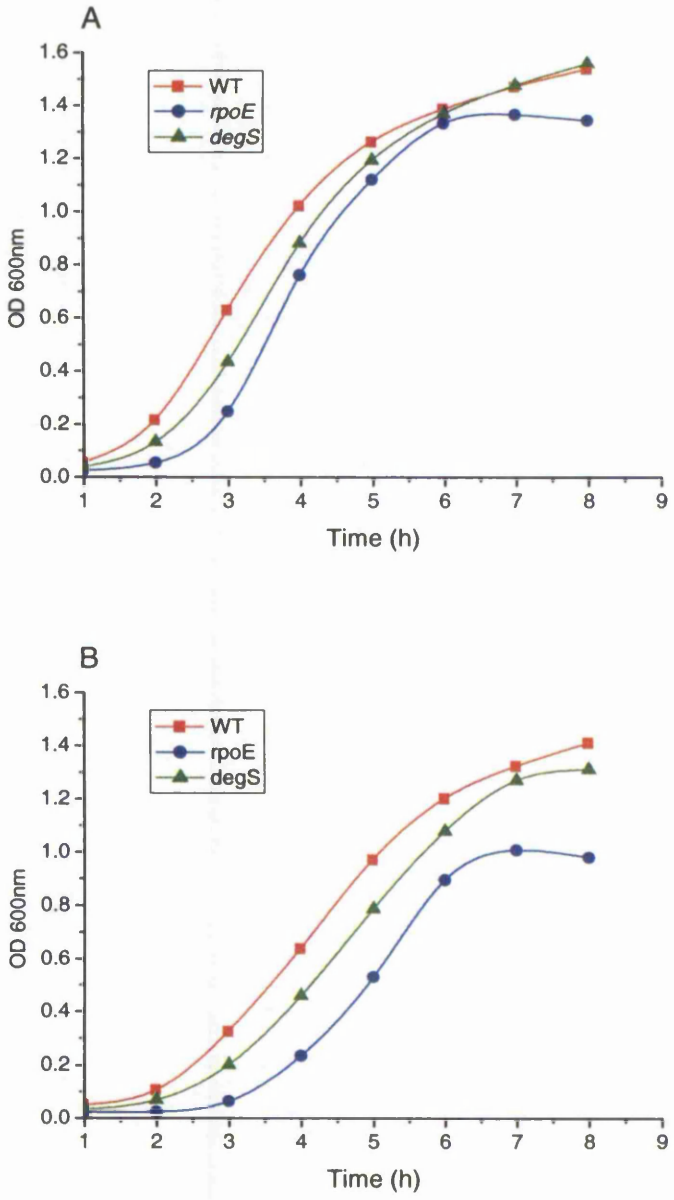
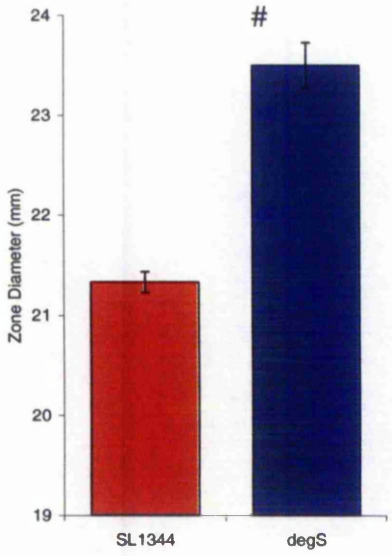


Figure 22. Effect of the *degS* mutation on the growth of *S.* Typhimurium in liquid media.

The *degS* mutant was compared with the WT strain and the *rpoE* mutant for its ability to grow aerobically in 25 ml LB-broth cultures at (A) 37°C and (B) 37°C with 3% ethanol. Growth was monitored for 8 hours and measured spectrophotometrically at OD600nm.

A) 3% Hydrogen Peroxide



B) Polymixin B

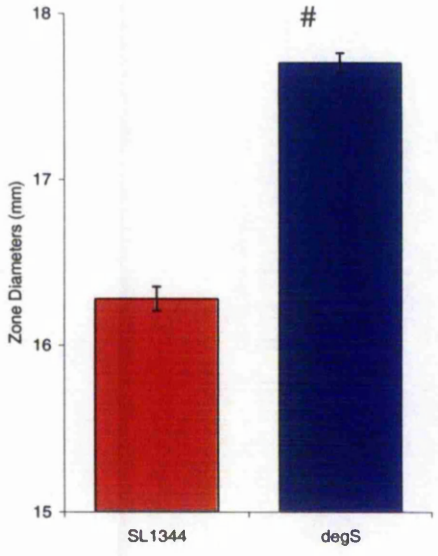


Figure 23. Sensitivity of a *S.* Typhimurium *degS* mutant to A) 3% hydrogen peroxide and B) 100U polymixin B.

The *degS* mutant and WT strains were tested for their sensitivity to both 3% hydrogen peroxide and 100U of the antimicrobial peptide polymixin B by a disk diffusion assay. Bars represent the mean diameters of the zone of inhibition (mm) of 16 replicates whilst the error bars represent the standard deviation of the mean. # indicates significant difference (Mann Whitney Test, $p<0.001$).

6.6 Intracellular Survival of a *S.* Typhimurium *degS* Mutant

Given that a *S.* Typhimurium *rpoE* mutant survives poorly in murine macrophages, we hypothesized that the intracellular environment would also be harsh for a *S.* Typhimurium *degS* mutant. At 3h post infection, significantly less ($p < 0.05$) of the *rpoE* and the *degS* mutants were present inside of RAW 264.7 cells than the WT strain (figure 24). The WT strain replicated inside RAW 264.7 cells between 3h and 24h post infection, in contrast, during the same period the intracellular numbers of the *degS* and *rpoE* mutants decreased greater than ten fold. This indicates that the *degS* mutant and the *rpoE* mutant (as previously shown) are killed within macrophages. There is no significant difference ($p < 0.05$) in the intracellular numbers of the *rpoE* and the *degS* mutant at 3 or 24h. Thus the *degS* and the *rpoE* mutant appear to behave identically within macrophages.

6.7 Analysis of *S.* Typhimurium *degS* in a Murine Model of Infection

σ^E is essential for *S.* Typhimurium to cause infection of mice by either the oral or parenteral routes of infection. We would expect therefore that *degS* would similarly play an important role in *S.* Typhimurium virulence during both the systemic and oral phases of infection of mice.

We initially compared the virulence of the WT and *degS* strains by competition assay, ~10^3 of both WT and *degS* strains were given in a mixed infection via the IP route to mice. A competitive index (CI) of ~ 1 indicates that the strains are of equal virulence. The CI for *degS* v WT is 0.006 indicating that the *degS* mutant is severely attenuated.

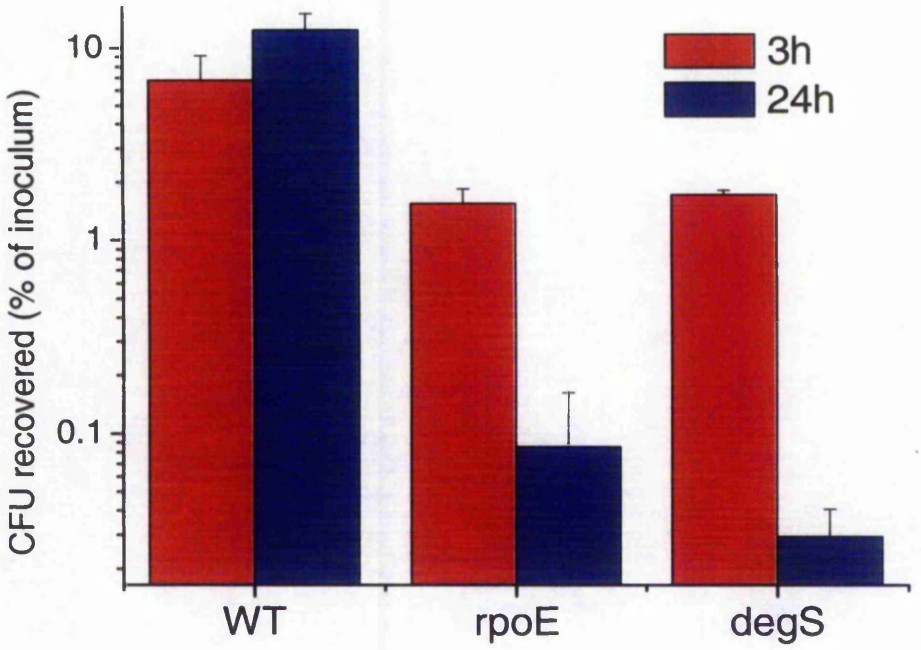


Figure 24-The affect of a *degS* mutation on *S.* Typhimurium invasion and survival in macrophages.

Bacteria at a multiplicity of infection of ~ 1:1 were incubated with the murine macrophage cell line RAW 264.7. The assay was performed as described in the text. The graphs show the number of viable bacteria (as a % of the initial inoculum) inside the macrophage at 3 h (white bars) and 24 h (black bars) after infection. Each bar represents the mean from triplicate experiments and the error bar indicates SD.

When a similar experiment was performed with the *S.* Typhimurium *rpoE* mutant v wild type *S.* Typhimurium, strain SL1344, the CI could not be determined accurately because no CFU of the *rpoE* mutant could be isolated from either the liver or the spleen (M Roberts, pers. comm.).

The *rpoE* and *degS* strains possess the same selectable marker (Km^R), therefore it is not possible to compare the virulence of the *rpoE* and *degS* mutants using the standard competition assay. Instead we gave single doses of $1x10^5$ CFU of the *rpoE* or *degS* mutant individually via the IP route to groups of five mice and enumerated the number of each strain present in the spleen and liver of individual mice 24h later (Figure 25). Bacteria could be isolated from the spleens and livers of all of the mice infected with the *degS* mutant. The numbers of *degS* bacteria isolated ranged from ~ 100 to 2000 CFU. In contrast, the *rpoE* mutant was only isolated from one of the organs (the spleen) from one of the mice. All of the parenteral infection studies indicate that the *degS* mutant is less severely attenuated than the *rpoE* mutant.

We also investigated the role of DegS during infection via the oral route. Mice were inoculated orally with either $5x10^5$ CFU of the WT strain or $1.6 x10^8$ CFU of the *degS* mutant and the number of bacteria present in specific organs was determined 7d later (figure 26). Even though the dose of the *degS* mutant was much higher than the WT strain there were significantly less ($p < 0.05$) *degS* than WT bacteria in all organs examined. The most dramatic difference can be seen in the liver and spleen where the numbers of WT bacteria isolated is ~ 10^6 fold greater than that seen with the *degS* strain.

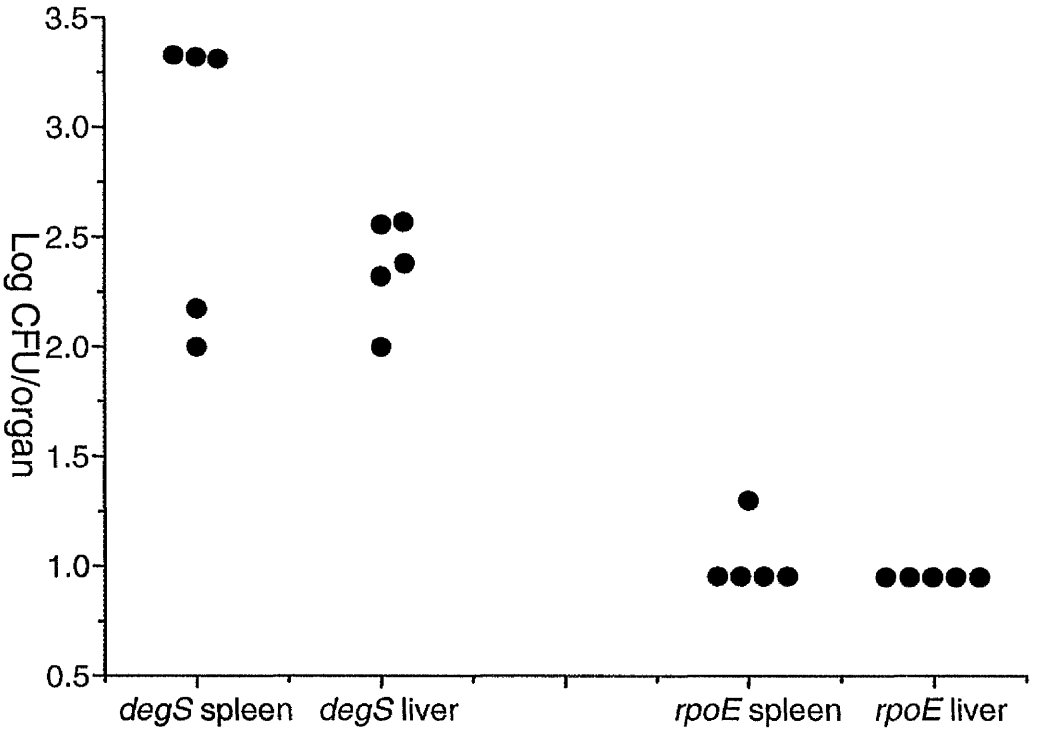


Figure 25. A comparison of the ability of *S.* Typhimurium *degS* and *rpoE* mutants to survive *in vivo* in murine organs following parenteral administration.

Single doses of 1x 10^5 CFU of the *rpoE* or *degS* mutant were given individually via the IP route to groups of five female BALB/c mice. After 24 h bacteria in the spleens and livers were enumerated. Each filled circle represents the count from the organ of an individual mouse.

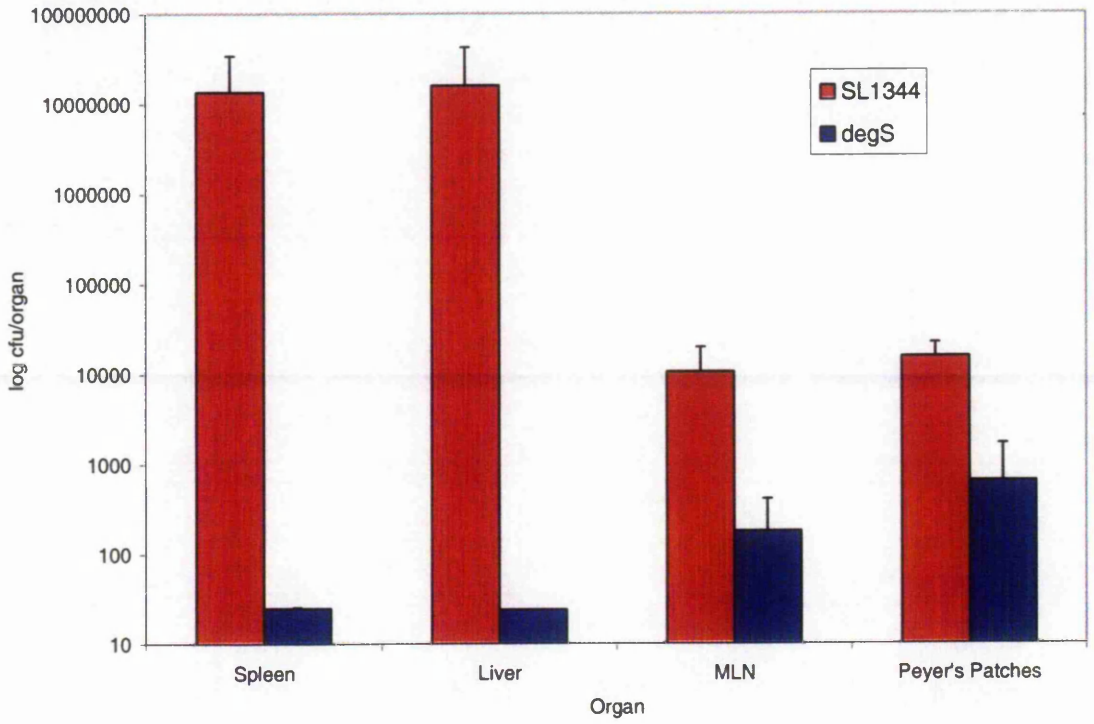


Figure 26. Effect of the *degS* mutation on the ability of *S.* Typhimurium infect mice via the oral route.

Mice were inoculated orally with either $5x10^5$ CFU of the WT strain or $1.6 x10^8$ CFU of the *degS* mutant and the number of bacteria present in different organs determined 7d later. The bar represents the mean of 4 mice and the error bar indicates the SD. MLN, mesenteric lymph node.

6.8 Discussion

We have previously shown that *S.* Typhimurium σ^E is important against a variety of stress and is critically important for survival of *S.* Typhimurium *in vivo*. The majority of the research on the activation and regulation of the σ^E pathway has been carried out on laboratory strains of *E. coli*. The number of genes and the organization of the *rpoE* operons of *E. coli* and *S.* Typhimurium are identical. However, there are differences in the regulation and importance of σ^E to the two organisms. For example, σ^E is essential for the viability of laboratory strains of *E. coli* at all temperatures and members of the σ^E regulon are necessary for the growth of *E. coli* at 42°C, neither is the case for *S.* Typhimurium (Hiratsu *et al.*, 1995;De Las, Connolly, and Gross, 1997a;Humphreys *et al.*, 1999).

DegS, a serine protease, is reported to be essential for σ^E activation and cell viability in *E. coli* (Alba *et al.*, 2001). Recently, *degS* was identified as a gene important for the ability of a clinical isolate of *E. coli* to cause extraintestinal infections in mice (Redford, Roesch, and Welch, 2003). *E. coli* is an extracellular pathogen in contrast to *S.* Typhimurium which is an intracellular pathogen. In view of the difference in the pathogenesis of *S.* Typhimurium and *E. coli* infections and the role of σ^E in the physiology of *E. coli* and *S.* Typhimurium we were interested in examining the importance of *degS* to *S.* Typhimurium *in vitro* and *in vivo*.

Although *degS* is reported to be essential for the viability of laboratory strains of *E. coli* under a variety of temperatures, its importance depends on the genetic background of the strain. In particular *degS* was more important for strains that also possessed other mutations suach as Δ*ompR*. (Alba *et al.*, 2001). Laboratory strains of *E. coli* with

mutations in *degS* are thought to be able to be isolated due to secondary suppressor mutations in one or more unknown genes (Alba *et al.*, 2001). These strains have a small colony phenotype (Bass, Gu, and Christen, 1996;Waller and Sauer, 1996;Alba *et al.*, 2001). In contrast a *degS* mutant of a clinical *E. coli* strain (CFT073) did not have a small colony phenotype, although the colonies were more translucent (Redford, Roesch, and Welch, 2003). DegS did not appear to be essential for the viability of CFT073 and the *degS* strain grew normally in LB at 37°C (Redford, Roesch, and Welch, 2003). However, in more stressful environments (43°C and 3% ethanol) growth of the *degS* mutant was severely affected (Redford, Roesch, and Welch, 2003).

As with *rpoE*, *degS* does not appear to be essential for *S.* Typhimurium under standard growth conditions *in vitro*. The colony morphology of the *S.* Typhimurium *degS* strain was identical to its WT parent on LB agar plates. At 37°C in LB broth the *degS* mutant demonstrates an extended lag phase in comparison to the WT strain but this is not as protracted as that of the *rpoE* mutant. The growth patterns of the 3 strains were the same at 43°C (GR, data not shown). In the presence of 3% ethanol, we find that the WT, *rpoE* and *degS* strains all have reduced growth rates, but display similar kinetics in relation to each other as they do at 37°C, the only difference being that the *rpoE* mutant enters stationary phase at a lower OD600nm than the other two strains. Thus in terms of growth the *degS* mutant of *S.* Typhimurium does not behave identically to an *rpoE* mutant. The effect of inactivation of *degS* on growth at elevated temperature and in the presence of ethanol is much smaller for *S.* Typhimurium than a clinical strain of *E. coli*.

The difference in the growth kinetics of the *degS* and *rpoE S.* Typhimurium mutant strains suggests that there may be active (free) σ^E present in the *degS* mutant strain. This

is supported by the finding that the *rpoE*P3::*lacZ* reporter gene is ~ 2X more active in the *degS* mutant than the *rpoE* mutant.

The *degS* mutation significantly reduced the ability of *S.* Typhimurium to replicate and/or survive intracellular within macrophages. There was no significant difference in the intracellular behaviour of the *degS* and *rpoE* mutants. This was the only assay (including sensitivity to hydrogen peroxide and polymixin B, GR data not shown) in which a difference between the *S.* Typhimurium *rpoE* and *degS* mutants was not found. There maybe a number of reasons for this, it maybe that the resolving power of the assay is not sufficient to differentiate between the two mutants. Alternatively, it maybe that if there is a *degS* –independent pathway for activation of the σ^E-pathway (see below) this is not activated by the intracellular environment of Raw 264.7 cells *in vitro.*

If the ability to survive within the macrophage *in vitro* is reflective upon the degree of virulence observed within a mouse model (Baumler *et al.*, 1994), we would have expected the *degS* and *rpoE* mutants to behave similarly *in vivo.* The *S.* Typhimurium *rpoE* mutant used in this study (GVB311) is severely attenuated in a mouse model via both the oral and parenteral infection routes, and provides no protection when used as a live vaccine strain against wild type *S.* Typhimurium (Humphreys *et al.*, 1999). The *degS* mutant is also severely attenuated although less so than the *rpoE* mutant. When a mixed inoculum containing ~ 1 x 10^3 CFU of both the WT and *degS* strains was administered to mice IP we could still isolate ~5x10^2 CFU of the *degS* mutant from both livers and spleens 3 days post infection. In contrast when a similar experiment was performed with the *rpoE* mutant v WT strain no CFU of the *rpoE* mutant could be isolated at day 3 (the level of detection was 10 CFU/organ).

When a higher dose (1 x 10^5) of the *rpoE* or *degS* mutants was given to mice IP, both of the strains were unable to establish infection in the liver and spleen and were cleared rapidly. However, whereas *degS* bacteria could be isolated from the liver and spleens of all infected mice, the *rpoE* mutant could only be isolated (in low numbers) from the spleen of one of five animals 24h after challenge. Therefore, the *degS* mutant survived at least 100 to 1000-fold better than the *rpoE* mutant.

The ability of the *degS* mutant to infect mice via the oral route was compared with the WT strain. Higher numbers of WT bacteria than *degS* were found in all organs at 7 d post-infection despite mice receiving ~ a 300-fold lower dose of the WT strain. The WT strain was found in higher numbers at system sites (liver and spleen) than at gut-associated sites (PP and MLN). The pattern is reversed for the *degS* mutant with more bacteria recovered from the PP and MLN than liver and spleen. Interestingly this is also the case for *S.* Typhimurium strains with mutations in *rpoE* and the σ^E-regulated genes *htrA* and *surA* (Humphreys *et al.*, 1999;Sydenham *et al.*, 2000). This indicates that either that σ^E-regulated genes are important for translocation of *S.* Typhimurium to systemic sites from the gut- associated lymphoid tissue and/or that σ^E-regulated genes are more important for survival at systemic sites. As *degS* and *rpoE* mutants are highly attenuated when administered parenterally it suggests that the latter is more likely.

In all of our studies, except the macrophage infection assay, inactivation of *degS* had a less severe effect on *S.* Typhimurium than inactivation of *rpoE*. This indicates that the σ^E pathway can be activated in a *degS* -independent manner, although not to the extent seen in a DegS -dependent manner. It may be that in certain situations YaeL can cleave RseA and liberate σ^E in the absence of DegS. The PDZ domains of both DegS and YaeL negatively regulate their proteolytic activity against RseA (Alba and Gross,

2004). In *E. coli*, YaeL lacking the PDZ domain can activate the σ^E pathway in the absence of DegS (Kanehara, Ito, and Akiyama, 2003;Bohn, Collier, and Bouloc, 2004). In fact, activation by YaeLΔPDZ is more efficient in a *degS* than a wild type background (Bohn, Collier, and Bouloc, 2004). Therefore, in the *S.* Typhimurium *degS* strain signals that interact with the PDZ domain of YaeL could lead to activation of σ^E. Interestingly, free σ^E present within *S.* Typhimurium *degS*, although only at a low level, is enough to partially correct some of the severe phenotypes observed with the *S.* Typhimurium *rpoE* mutant, including *in vivo* survival. We are currently investigating the DegS independent activation of the *S.* Typhimurium σ^E regulon.

The inability to construct a *S.* Typhimurium *yaeL* mutant may be due to a number of reasons; YaeL may possibly have a function outwith σ^E activation; disruption of *yaeL* may affect downstream genes which may be lethal such as *yaeT* (Knuth *et al.*, 2004). In *E. coli* another function for YaeL is not considered due to the requirement of functional σ^E for cell viability. However, we are certain that *S.* Typhimurium can survive in the absence of σ^E, which may imply another role for YaeL beyond σ^E activation. This should be investigated further by trying to mutate the CDS of *yaeL* with an additional copy of *yaeT* located on a plasmid.

Chapter 7 - Construction and Characterisation of *S.* Typhimurium Δ*sbmA* and Δ*yaiW* Mutants

7.1 Introduction

sbmA was first identified in the plant symbiont *Rhizobium meliloti* where it is known as *bacA*, and is predicted to encode a cytoplasmic membrane transport protein with seven transmembrane domains (Glazebrook, Ichige, and Walker, 1993). A *Rhizobium meliloti* (now *Sinorhizobium) sbmA* mutant strain is unable to form bacteroids within a plant root nodule and instead rapidly senesces. The amino acid sequence of *Sinorhizobium meliloti* SbmA is 64% identical to, 79% similar to and functionally interchangeable with the *E. coli* SbmA protein (Ichige and Walker, 1997). SbmA is thought to be a transporter of bleomycin, and microcins B17 and J25 (Lavina, Pugsley, and Moreno, 1986), due to the high level resistance to these agents observed in both *E. coli* and *Sinorhizobium* mutants. However, the true function of SbmA still remains unknown, but a deletion of *E. coli sbmA* has no effect on cell growth and is therefore not critical for viability. Homologues of SbmA have also been reported in a variety of Gram-negative bacteria such as *Brucella abortus*, *Klebsiella pneumoniae*, *Shigella flexneri* and *Agrobacterium tumafaciens* (Yorgey and Kolter, 1993), and also within Gram-positive bacteria such as *Staphylococcus aureus* and *Nocardia antidiscavarium.* Gene conservation amongst bacteria which are not closely related to each other and are capable of surviving in a wide range of environments may be indicative of a function that confers a selective advantage. Yorgey and Kolter, 1993 have suggested that SbmA may function as an environmental sensor.

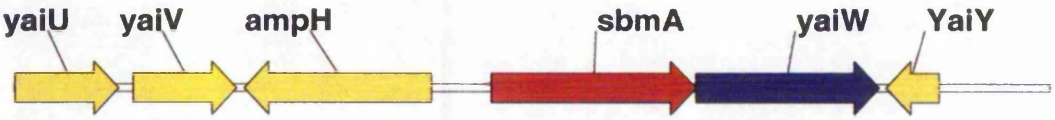
S. meliloti bacA mutants has now been well characterised. It appears that one of the roles of this gene may be to alter LPS fatty acid modifications (Roop *et al.*, 2002). This same group reports that the *bacA* mutant strain also has significantly less 27-OH-$C_{28:0}$, a very long chain fatty acid on lipid A (Kannenberg and Carlson, 2001), in comparison to

its wild type counterpart, and suggest that this may be the underlying reason for the mutants increased sensitivity to detergents, dyes and ethanol. This theory is based on the notion that changes in phospholipid affect acid resistance in other bacterial systems (Chang and Cronan, Jr., 1999). However, sensitivities to agents observed with the *S. meliloti sbmA* mutant have also been reported for the *E. coli sbmA* mutant (Ichige and Walker, 1997), but 27-OH-$C_{28:0}$ is absent from the LPS of enteric bacteria (Bhat *et al.*, 1991a;Bhat *et al.*, 1991b).

The *bacA* (*sbmA*) gene in *B. abortus* is critically required for the maintenance of chronic infection in a mouse model, with mutations resulting in an attenuated strain. This attenuation has been attributed to a loss of intracellular survival in host macrophages. However, such mutations are still capable of producing both specific cellular and humoral immune responses, and is currently under investigations as a possible vaccine candidate (LeVier *et al.*, 2000;Roop *et al.*, 2002). Interestingly although the function of *sbmA* has been evaluated in a number of bacterial species, none of the investigators have commented on the downstream gene, *yaiW*, which appears to be transcriptionally linked with *sbmA* if operon prediction analysis is performed (2004). Does a mutation in *sbmA* have a polar effect on yaiW?

Thus far little work had been carried out regarding the regulation of *sbmA/bacA*, other than that under standard laboratory growth conditions little expression is observed. In this chapter we investigate the virulence properties of a *S.* Typhimurium *sbmA* mutant and address the regulation of this gene, as well as investigating any effects a mutation in *sbmA* may have on *yaiW* in *S.* Typhimurium.

7.2 Genetic Locale of the *sbmA* Operon in S. Typhimurium.



The *sbmA* operon is located at 8.5 minutes on the *S.* Typhimurium genetic map and is divergently expressed in relation to both upstream and downstream neighbouring genes.

7.3 RpoE Regulation of *sbmA*

Although the functional role of the *sbmA* gene product has been investigated in a number of bacterial species the regulation of this gene has been overlooked, other than that when cultured under standard growth conditions *sbmA* in *E. coli* is reported to be poorly transcribed (Lavina, Pugsley, and Moreno, 1986).

It was hypothesised that σ^E regulates *sbmA* in *S.* Typhimurium because this gene and its transcriptional partner, *yaiW*, were identified in a two plasmid screen for σ^E regulated genes in both *S.* Typhimurium and *E. coli* (Rezuchova *et al.*, 2003) and because they were 4 fold and 3 fold upregulated respectively on our microarray analysis after overexpression of σ^E. We constructed a *sbmA* promoter fusion (*sbmA*P) of the entire intergenic region between *ampH* and *sbmA* with *lacZ*, which consists of 500bp of sequence upstream of the predicted start codon of *sbmA*, and includes a putative σ^E dependent promoter region predicted by both a promoter consensus search of the *S.* Typhimurium genome and S1 nuclease mapping.

As transcriptional regulation of the *sbmA* operon remains unconfirmed in any organism, we looked at the transcriptional activity of the predicted regulatory region in a variety of

genetic backgrounds. These included 1) the *surA* mutant strain, already shown to induce the autoregulated *rpoEP3* promoter. 2) An *rpoE* mutant to investigate if σ^E is indeed a regulatory factor of this operon. 3) A *cpxA* mutant to see if *sbmA* belongs to the subset of genes like *htrA* which are co-regulated by both CpxA and σ^E 4) *rpoN* and *rpoS* backgrounds to see if other alternative sigma factors control *sbmA* expression and finally 5) in an *sbmA* mutant to see if there is any autoregulation of the operon by SbmA itself.

As figure 27 shows, activity of the *sbmAP* is relatively low in wild type *S.* Typhimurium considering the predicted entire regulatory region of *sbmA* has been fused with *lacZ*. At the same phases of growth as those depicted for *sbmAP*, activity of the complete regulatory region of *rpoH* (rpoHpall) was 2.5 fold higher after 3 hours and 10 fold greater after 6 hours of growth. This low level transcription is in agreement with *sbmA* mRNA transcript levels measured in a wild type *E. coli* background (Lavina, Pugsley, and Moreno, 1986), and confirms the reliability and viability of our promoter fusion. However, a 2 fold increase can be observed between mid exponential phase and stationary phase, although a greater fold induction is usually seen for stationary phase induction of σ^E regulated promoters, with the *rpoEP3* promoter induced nearly 5 fold upon entry into stationary phase. The increase in *lacZ* levels seen could be attributed to a change in the growth phase of the cells (Warner and Lolkema, 2002) rather than an induction caused by the elevated levels of σ^E in stationary phase. However, as *sbmA* was identified as putatively σ^E regulated through overexpression of *rpoE* we doubt that this is the case, and *sbmA* does indeed possess a σ^E regulated promoter. This correlates with the activity of the promoter in a *surA* mutant background. Here an 8 fold and 9 fold

induction in activity can be measured after 3 and 6 hours respectively, indicative of the presence of a σ^E dependent promoter. This is also confirmed by the increased activity of

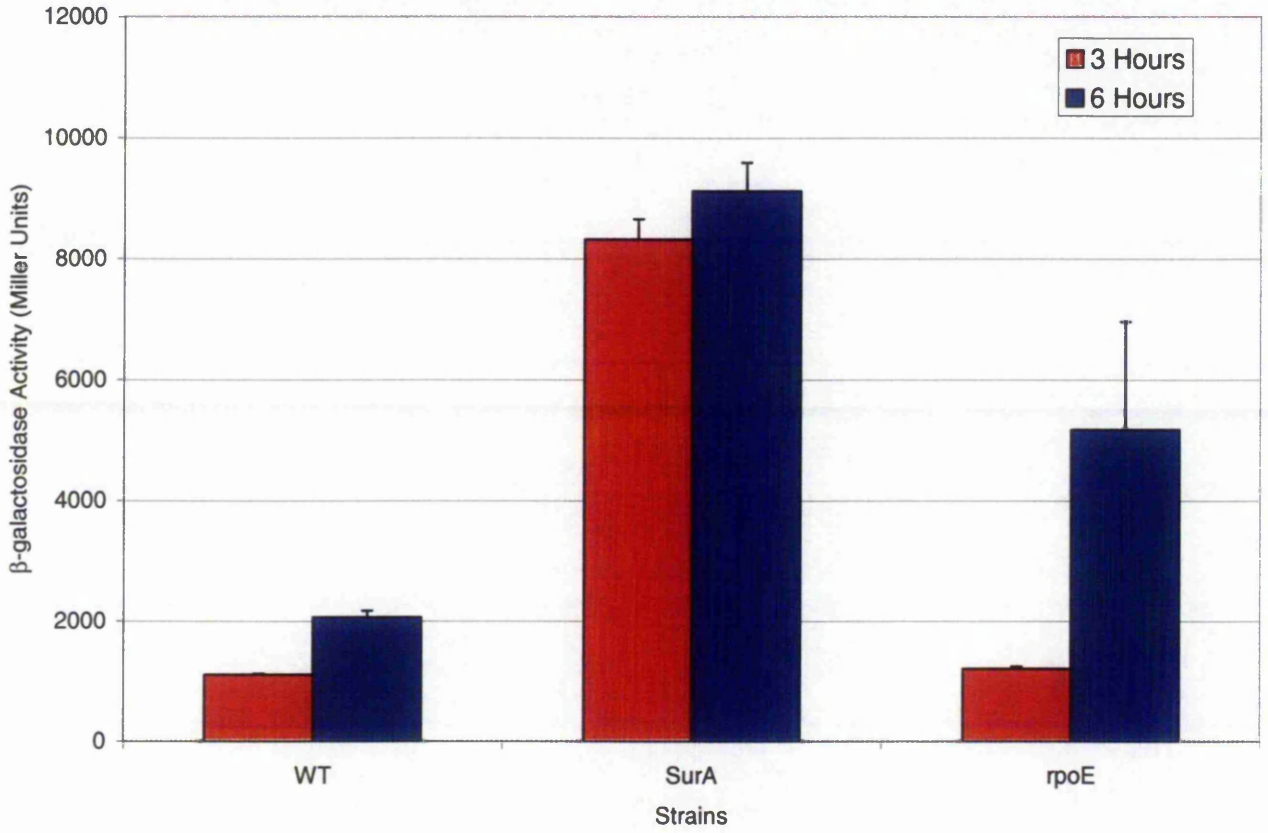
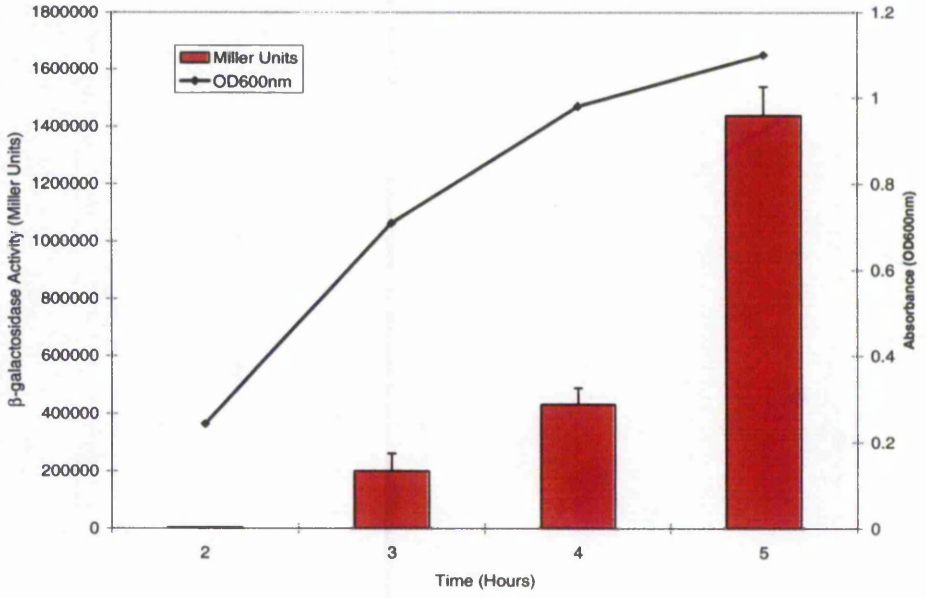


Figure 27. Regulation of the *sbmA* operon as determined by β-galactosidase assays performed as described in chapter 2 and 4. Promoter activity was assayed during mid exponential and stationary phase in a variety of genetic backgrounds including those known to induce the σ^E regulon.

the *sbmAP* after ovexpression of σ^E (figure 28) and confirms the result obtained from the microarrays. In the presence of 0.2% arabinose there is a 200 fold increase in *sbmAP* activity in comparison with the culture containing the P_{BAD} repressor glucose. Whether directly, or indirectly, *sbmA* is regulated by σ^E.

The most interesting result and probably the result that generates more questions than answers is the activity of the *sbmAP* in the absence of σ^E. At the 3 hour time point the activity of the promoter is almost identical to that in the wild type strain, but after 6 hours the promoter activity increases almost 7 fold. A number of hypotheses can be made to explain this result. The absence of σ^E may generate a cellular environment that requires an increase in functional SbmA/YaiW to restore homeostasis in a non- σ^E dependent fashion. σ^E itself may act as a repressor for another regulator of sbmA, or *sbmA* may also be regulated by another regulator such as σ^H, which has compensatory function for the absence of σ^E.

A)



B)

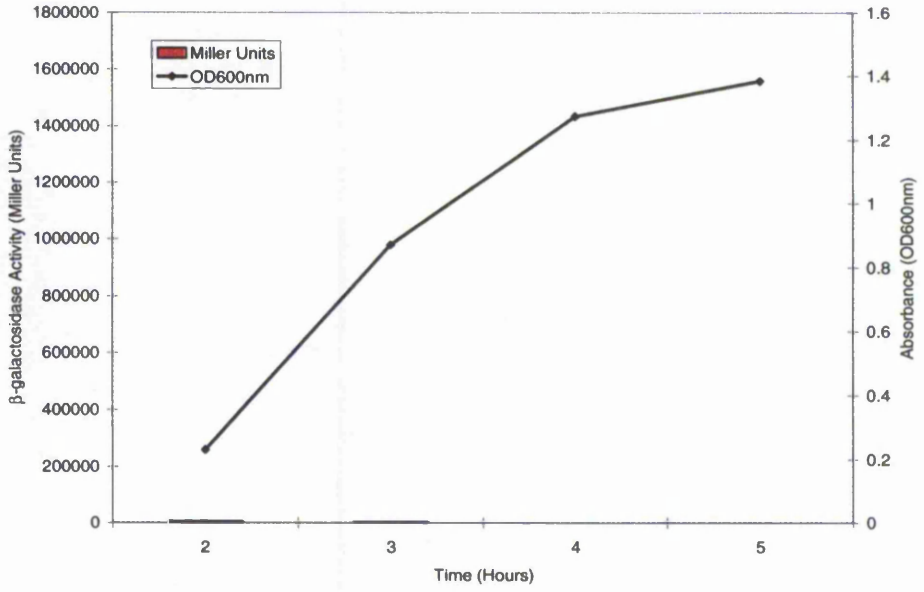


Figure 28. Effects of σ^E overexpression on the *sbmAP*.

As described in chapter 4, we used pAC-rpoEST4 to over-express σ^E in an arabinose dependent manner. **A** shows activity of *sbmaP* in the presence of arabinose, whilst **B** shows the same construct in the presence of the P_{BAD} repressor glucose. The growth curve of this experiment is also presented.

7.4 Characterisation of a *S.* Typhimurium *sbmA* Mutant Strain

As described in chapter 3 *sbmA* was one of the first genes we tried to mutate using λ Red mutagenesis. Using primers sbmARedF and sbmARedR, we managed to successfully delete the entire CDS of *sbmA* and replace it with a kanamycin cassette from plasmid pKD4, without removing any of the CDS of the downstream gene in the operon *yaiW*, or without theoretically disturbing the predicted operon regulatory region. The following section describes the characterisation of this mutant.

7.4.1 Growth Kinetics

Figure 29 overleaf depicts the growth of a *sbmA* mutant at 37°C in relation to its parent SL1344 strain and an isogenic *S.* Typhimurium *rpoE* mutant. This clearly demonstrates the extended lag phase exhibited by an *rpoE* mutant in L-broth without additional glucose as a carbon source, and illustrates that a *sbmA* mutant does not possess this phenotype, and in fact grows equally as well as the wild type strain under these conditions. In fact we were unable to detect a difference in growth between wild type *S.* Typhimurium and a *sbmA* mutant under all liquid growth conditions tested. This includes an increase in temperature to 42°C and 46°C and the addition of 3% ethanol to the culture medium. Increased temperature and 3% ethanol have been reported to impair growth of a *S. meliloti sbmA* mutant (Ichige and Walker, 1997), although this phenotype was not observed in the *E. coli sbmA* mutant.

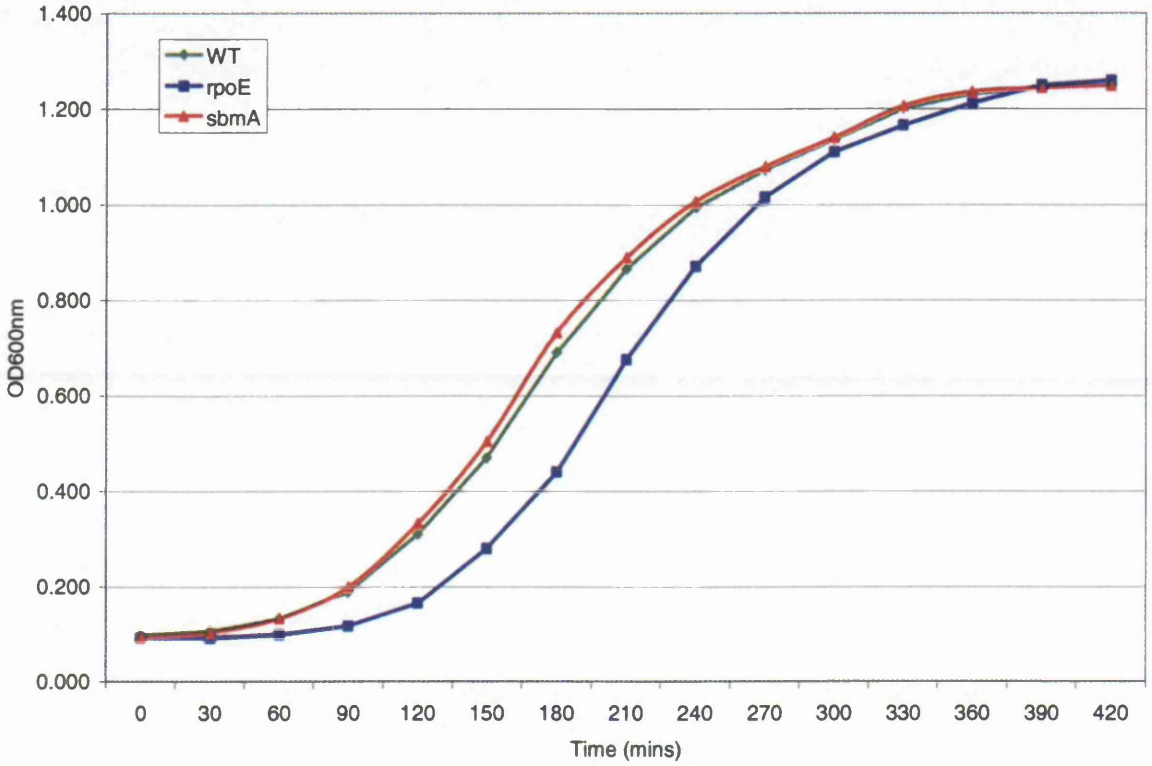


Figure 29. Comparative growth of SL1344 and isogenic *sbmA* and *rpoE* mutants in liquid media at 37°C.
This growth curve represents the mean of three independent experiments performed on the Bioscreen C machine. SEM have been calculated and plotted but are too small to be visible on the data points.

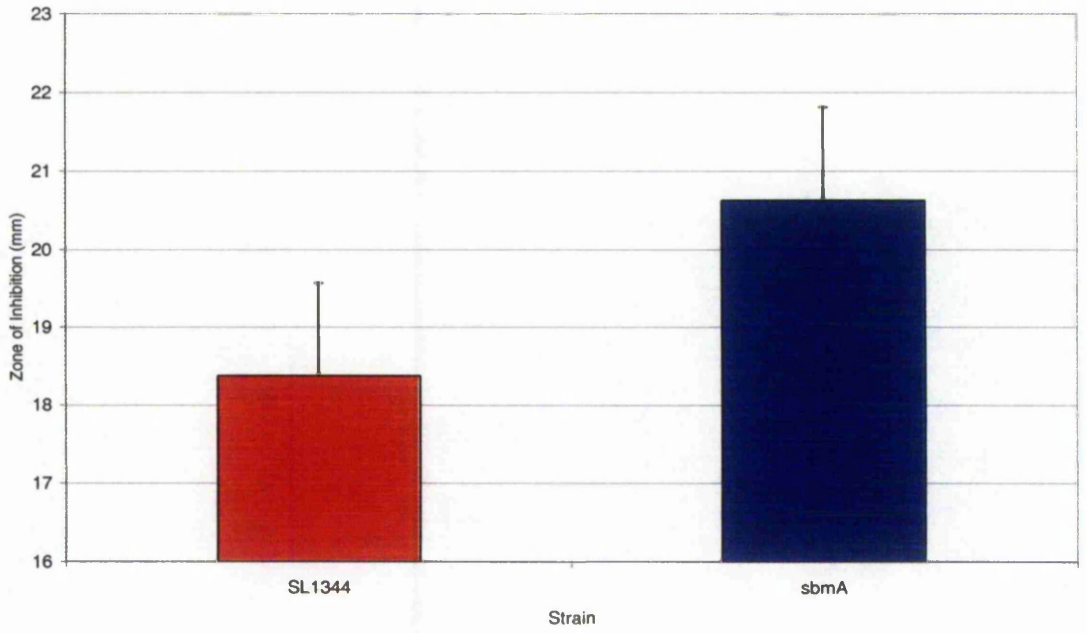
7.4.2 Disc Diffusion Assays

S. meliloti sbmA mutants are more resistant to a variety of agents including microcin J25, SDS, ethanol, bleomycin, and gentamicin (Ferguson, Roop, and Walker, 2002). An *E. coli sbmA* mutation did not affect resistance to ethanol, aminoglycosides or SDS (Ichige and Walker, 1997). We tested a number of these agents against the *S.* Typhimurium *sbmA* mutant, as well as agents to which the *S.* Typhimurium *rpoE* mutant has an increased sensitivity. These include the antimicrobial peptide polymixin B and the oxidative agents hydrogen peroxide and paraquat, which previous *sbmA* mutants have not been tested against.

Interestingly the *S.* Typhimurium Δ*sbmA* strain was significantly more sensitive to discs impregnated with both 3% hydrogen peroxide (figure 30a) ($P<0.05$, student t test) and 2mM paraquat than the control wild type strain. The *sbmA* mutation did not affect polymixin B, SDS, ethanol or bleomycin sensitivity of *S.* Typhimurium (GR, data not shown).

A comparison between resistance of wild type *S.* Typhimurium and Δ*sbmA* could not be made for microcin J25 as *S.* Typhimurium is intrinsically resistant to this peptide antibiotic (Vincent *et al.*, 2004). Comparison of the sensitivity to the aminoglycoside antibiotic gentamicin was made using disks containing 200μg gentamicin and a significant increased resistance ($p<0.05$, student t test) could be observed with on average a 3mm reduction to the zone of inhibition seen with the *sbmA* mutant in comparison to the wild type strain (figure 30b).

A) 3% hydrogen peroxide



B) 200µg gentamicin

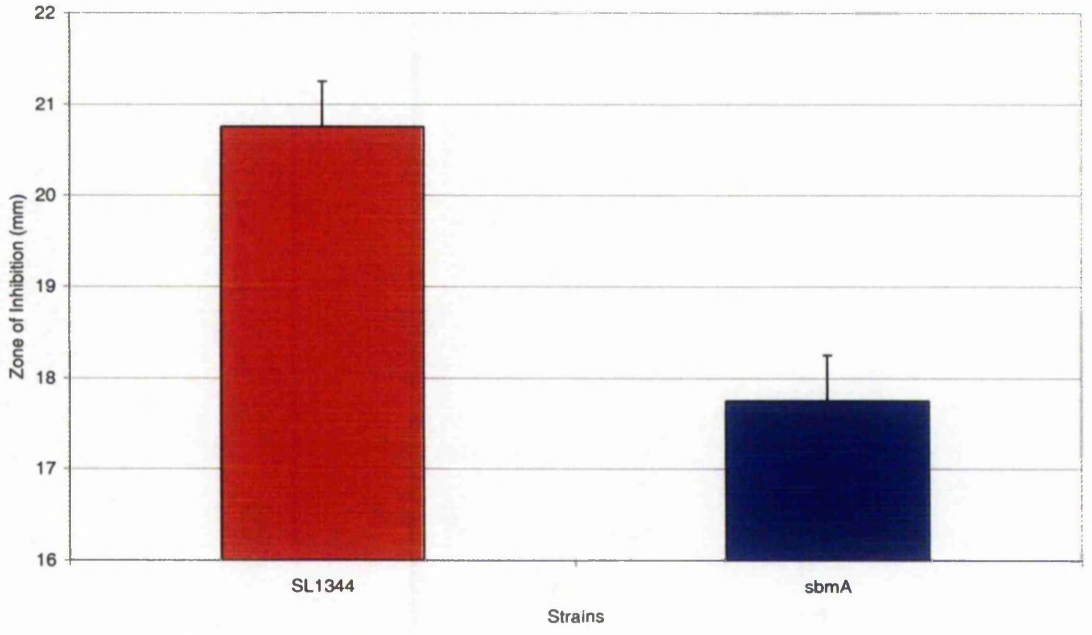


Figure 30. Sensitivity of a Δ*sbmA S.* Typhimurium strain to A) 3% hydrogen peroxide and B) 200µg gentamicin.
The *sbmA* mutant and WT strains were tested for their sensitivity to both 3% hydrogen peroxide and 200µg of the aminoglycoside gentamicin by a disk diffusion assay. Bars represent the mean diameters of the zone of inhibition (mm) of 16 replicates whilst the error bars represent the standard deviation of the mean.

7.4.3 Systemic Infection of Mice

A *sbmA* mutant of *Brucella abortus* was able to colonise the spleens and livers of IP experimentally infected BALB/c mice at similar levels to the wild type strain for the first two weeks post infection, but after this point the mutant was rapidly cleared from the mice (LeVier *et al.*, 2000). The same mutant had a decreased intracellular survival within a macrophage cell line 12 hours post infection and were almost completely eradicated by 24 hours. This reduced survival was proposed as the reason for its inability in maintaining a state of chronic infection.

From our knowledge of infection models of *S.* Typhimurium we would predict that any given mutant with such an inability to survive in macrophages would have a marked reduction in survival within a host in comparison to the wild type strain, as is the case with *rpoE* (Humphreys *et al.*, 1999), *htrA* (Chatfield *et al.*, 1992c), *degS* and many other *S.* Typhimurium mutants (Baumler *et al.*, 1994). After performing an invasion assay on RAW 264.7 murine macrophages with SL1344 and the *sbmA* mutant, equal CFU/ml could be isolated after both 3 and 24 hours post infection (GR, data not shown). This indicated that the *sbmA* mutant would not be particularly attenuated in a mouse model, as macrophage survival is often reflective of survival within the host (Baumler *et al.*, 1994).

We tried to establish whether a *S.* Typhimurium *sbmA* mutant had a similar phenotype to that described in *Brucella* regarding long term infection, and also investigated the numbers of colonising Δ*sbmA* and wild type strains over a shorter 3 day experiment. Using a competition assay, groups of 5 BALB/c mice were administered with 200µl of a mixed infection of wild type SL1344 and the *sbmA* mutant, comprising of ~10^3 CFU of each

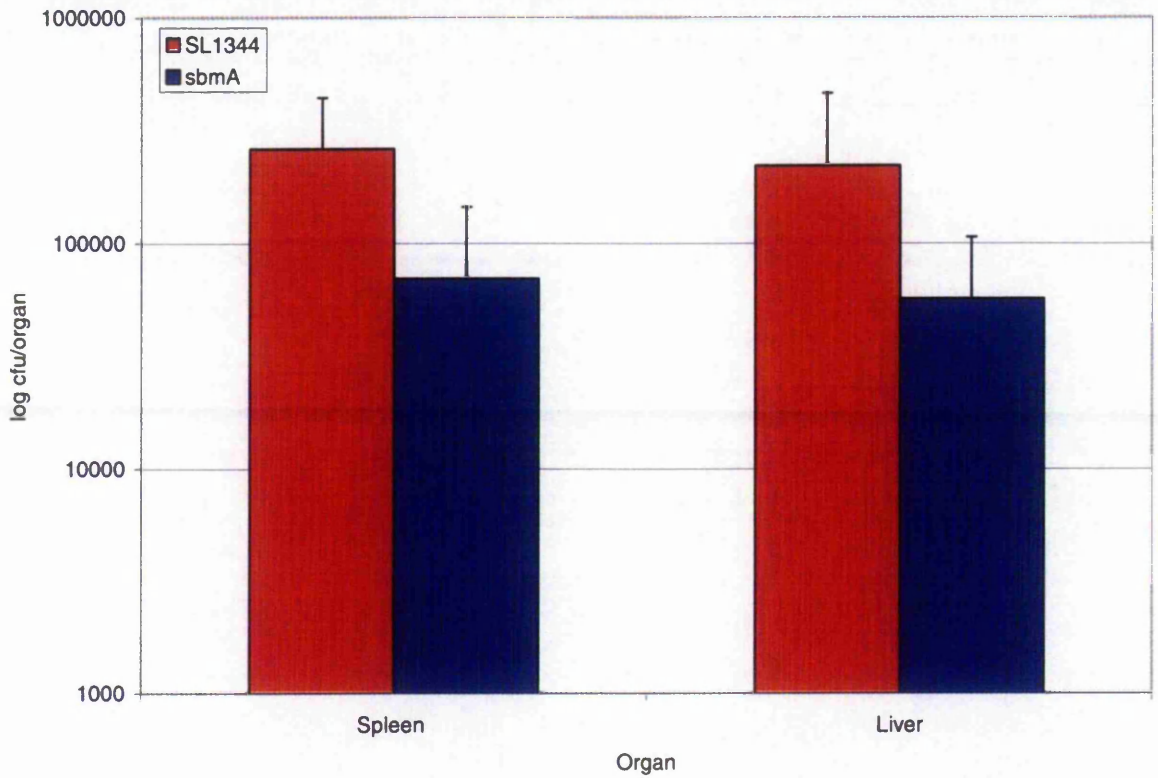


Figure 31. Intraperitoneal competition assay between wild type *S.* Typhimurium and an isogenic Δ*sbmA* mutant in BALB/c mice. A mixed dose of ~10^3 CFU/200µl was administered by the stated route. The log CFU/organ is a mean of 5 mice and the error bars represent standard deviation of the mean.

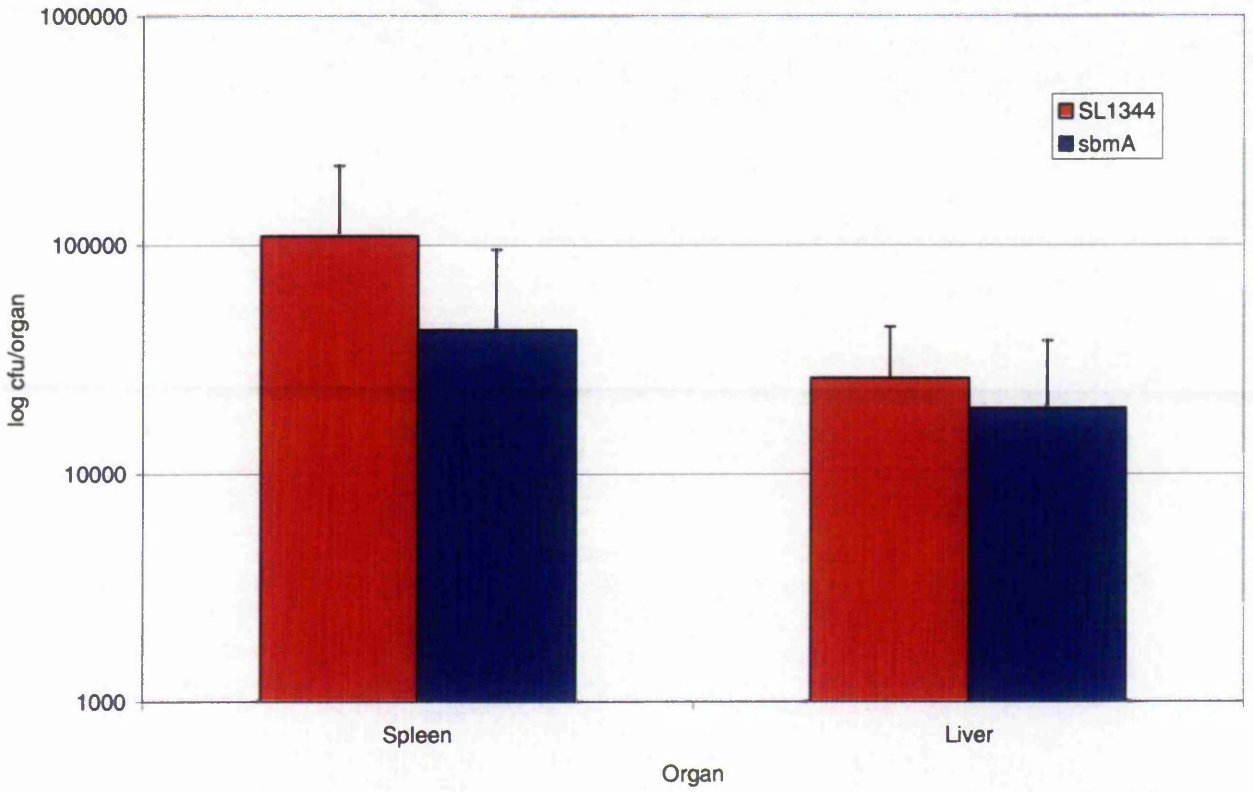


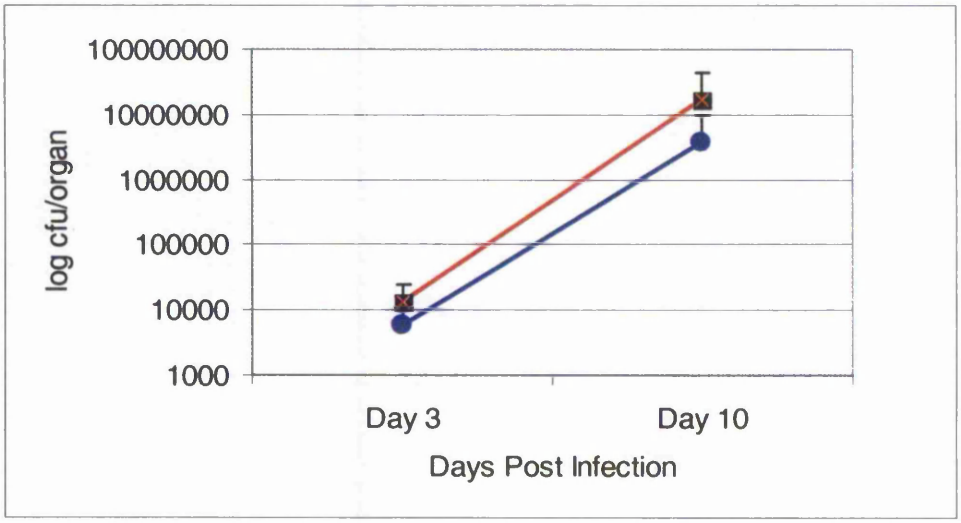
Figure 32. Intravenous competition assay between wild type *S.* Typhimurium and an isogenic Δ*sbmA* mutant in BALB/c mice. A mixed dose of ~10^3 CFU/200µl was administered by the stated route. The log CFU/organ is a mean of 5 mice and the error bars represent standard deviation of the mean.

strain per dose, via the IP and IV routes. Three days post infection the mice were euthanased and spleens and livers removed to estabilish CFU of each strain per organ. Figures 31 and 32 demonstrate the data obtained from these experiments. If the *S.* Typhimurium *ΔsbmA* mutant behaves similarly to that of the *Brucella* equivalent and the data obtained from the macrophage infections are accurate and reflective of virulence, then colonising numbers of the wild type strain and the *sbmA* mutant should be similar at this short time point. Via the IP route an average of approximately 2 x 10^5 CFU of SL1344 could be isolated from both the spleens and livers in comparison to approximately 7x10^4 CFU of the *sbmA* mutant. This equates to a competitive index of 0.5 and although reduced in comparison with the wild type strain, is not quite significantly different ($p>0.05$, student t test). A similar trend is seen after IV administration although numbers of bacteria isolated for both strains is reduced in comparison with IP, especially in the liver. This is a trend we have seen with other mutants including *htrA* (GR-data not shown), but we are unsure as to why this may be the case.

In order to try and investigate the possibility that *sbmA* is involved in persistent infection, we needed to use a different mouse model that would enable us to monitor bacterial numbers over a longer time period. To do this we used DBA/2 mice which have the same MHC haplotype as BALB/c but are ity^R and are therefore more resistant to *S.* Typhimurium infection (Robson and Vas, 1972;Plant and Glynn, 1974). We again utilised a competition assay to IP deliver a similar dose, ~10^3 CFU/dose, to that used for the infection of BALB/c mice via this same route. This time groups of 5 DBA/2 mice were inoculated and were to be euthanized 3 days post infection, so that a comparison between DBA/2 and BALB/c mice could be made, and 14 days post infection to see if like in *Brucella* the *sbmA* mutant is cleared 2 weeks after infection. Unfortunately

although more resistant to *S.* Typhimurium infection, the DBA/2 mice had to be culled on day 10 in accordance with the project licence guidelines, as they possessed a staring coat, were immobile, and had lost appetite. Figure 33 shows the data obtained from days 3 and 10. At the early time point the *ΔsbmA* had a CI~0.5 in the spleen, although equal numbers of the wild type strain and the *sbmA* mutant were isolated from the liver (CI~1.0). In comparison with the same time point for BALB/c mice the CFU/organ isolated from DBA/2 mice is nearly a log less for both strains. On day 10 the CFU/organ for the wild type strain had increased by 3 logs, but the *sbmA* mutant had not increased as much during these 7 days, with on average 20% less CFU/organ for both the spleen and liver, and is equivalent to a competition index of 0.2. However, although significantly less numbers of *ΔsbmA* were isolated from both the livers and spleens compared with WT ($p<0.05$, student t test), this still corresponds to greater than 10^6 CFU/organ for the *sbmA* mutant. To try and extend the experiment length we gave single infections of *ΔsbmA* to DBA/2 mice but the experiment again had to be terminated at the same time point as with the competition assay due to the condition of the mice.

A) Spleen



B) Liver

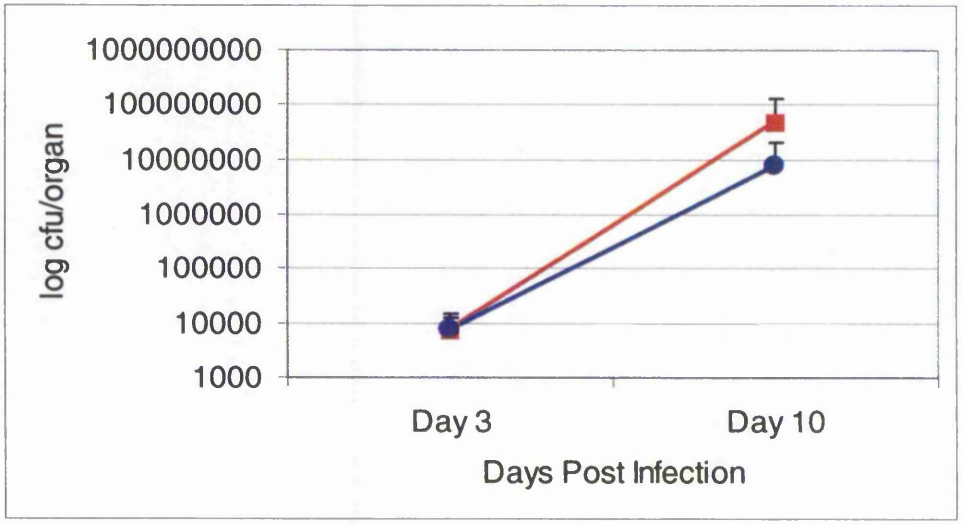


Figure 33. Intraperitoneal infection of DBA/2 mice with a mixed infection of wild type *S.* Typhimurium (red) and an isogenic Δ*sbmA* mutant (blue).
A mixed dose of ~10^3 CFU/200µl was administered by the stated route. The log CFU/organ is a mean of 5 mice and the error bars represent standard deviation of the mean.

7.4.4 Oral Infection of Mice

Single infections of $5x10^5$ CFU per 200µl dose were administered by oral gavage to groups of 5 BALB/c mice for both the wild type strain and the *sbmA* mutant. Mice were culled on days 5 and 7 post infection and bacteria isolated from the spleen, liver, Peyer's patches and mesenteric lymph nodes. Figure 34 illustrates a time course of this data. On day 5, 10 fold greater numbers of the wild type strain were isolated from both the spleen and liver in comparison with the *sbmA* mutant, with $3.1x10^5$ CFU/organ of wild type strain versus $3.9x10^4$ CFU/organ of the Δ*sbmA* mutant and $2.5x10^5$ CFU/organ of wild type strain versus $3.9x10^4$ CFU/organ of the Δ*sbmA* mutant for the spleen and liver respectively. Equivocal numbers, $6x10^3$, of the wild type strain and the Δ*sbmA* mutant were isolated from the MLN, and 2 fold greater of the Δ*sbmA* mutant than the wild type strain were isolated from the PP, $3x10^3$ versus $1.4x10^3$. By day 7, the patterns observed on day 5 were still present, other than that greater numbers of Δ*sbmA* were now present in the MLN as well as the PP relative to wild type *S.* Typhimurium numbers, with 6x 10^4 CFU of Δ*sbmA* versus $1x10^4$ CFU of the wild type strain, and $4.8x10^4$ CFU of Δ*sbmA* versus $1.5x10^4$ CFU of the wild type strain, for the MLN and PP respectively. Numbers in the spleen and liver had increased by 1.5 log for both the wild type strain and *sbmA* mutant, with ~10^6 CFU/organ isolated for Δ*sbmA* and ~10^7 CFU/organ for the wild type strain.

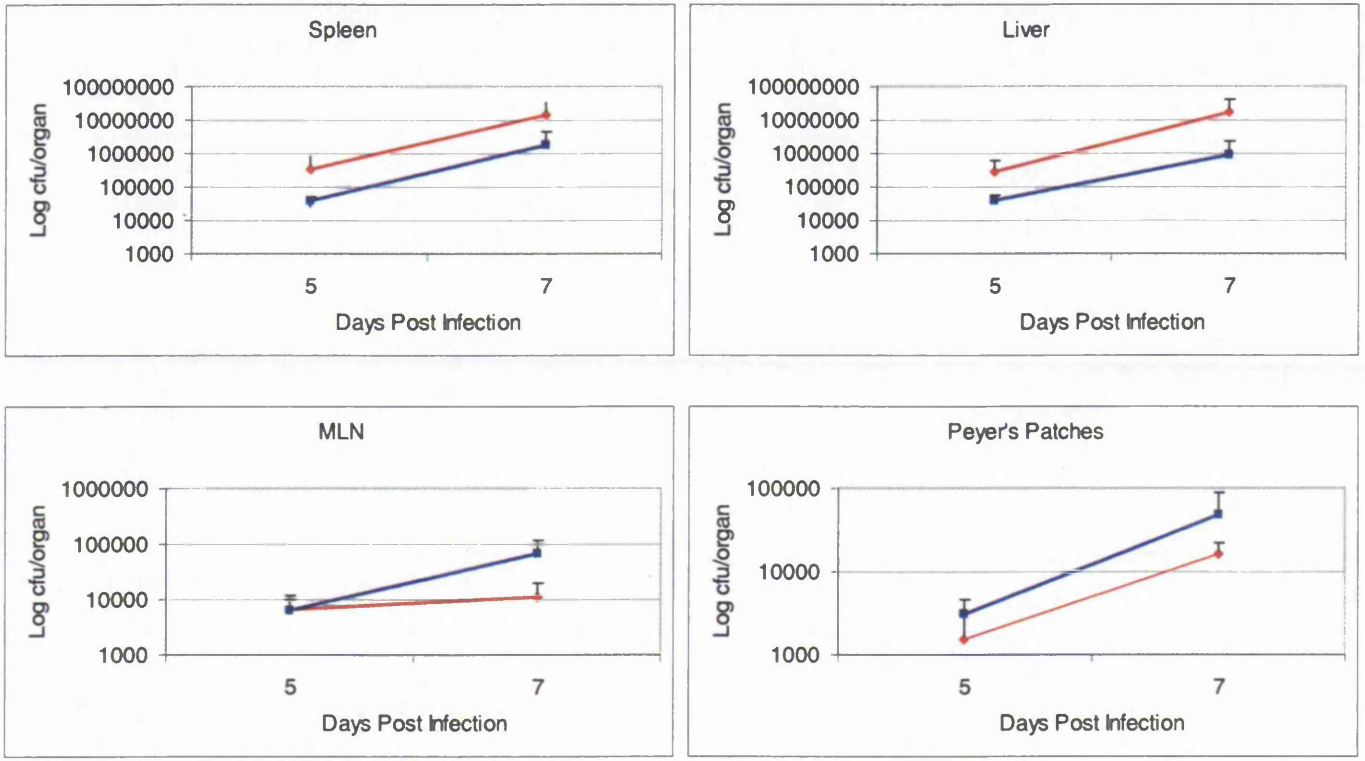


Figure 34. Oral Infection of BALB/c mice with wild type *S.* Typhimurium (red), and an isogenic Δ*sbmA* mutant (blue). Single doses of ~$5x10^5$ CFU/200µl were administered by oral gavage and CFU/organ established at 5 and 7 days post infection. Data presented is a mean of 5 mice and error bars represent the standard deviation of the mean.

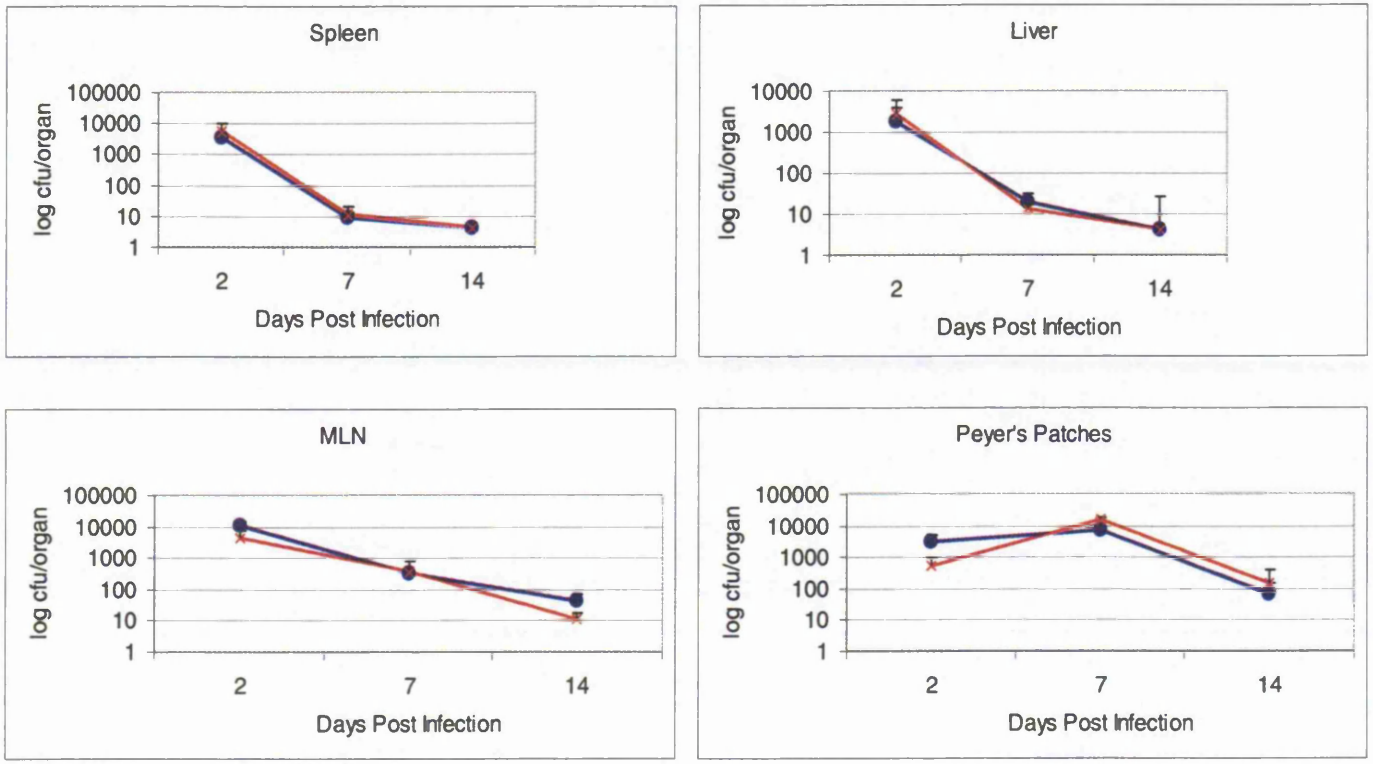


Figure 35. Oral infection of BALB/c mice with BRD915 Δ*sbmA* double mutant (red) and BRD915 (blue).
Approximately ~10^7 CFU per 200µl dose was administered by oral gavage and mice culled on days 2, 7 and 14 post infection. Data presented represents a mean of 5 mice and error bars are the standard deviation from the mean.

Δ*sbmA* as a *S.* Typhimurium vaccine strain in its own right is a poor candidate. We therefore investigated the possibility of combining Δ*sbmA* with an *htrA* mutant (BRD915) to see if their combined effect was more attenuated than the single *htrA* mutant. We performed single infections via oral gavage using ~10^7 CFU/200μl dose for each strain. Figure 35 illustrates the results from this experiment, and essentially the Δ*sbmA* Δ*htrA* double mutant behaved identically to the single *htrA* mutant, BRD915 with respect to CFU/organ.

7.5 Characterisation of a *S.* Typhimurium *yaiW* Mutant

As *sbmA* exists in an operon of two genes, with the second gene being *yaiW*, we were also interested to look at the effects of mutating this gene. Of all the work completed on *sbmA* so far, complementation of phenotypes observed with *sbmA* mutants has been difficult and none of the groups have investigated the possible polar effects on *yaiW*. This has not been a major problem for us in *S.* Typhimurium as there is limited phenotypic differences between the wild type strain and Δ*sbmA* strains, however the fact that there is a σ^E regulated promoter upstream of this operon could mean that YaiW plays an important role in the ESR and/or virulence in its own right. We constructed a Δ*yaiW* mutant using λ Red mutagenesis and linear DNA produced by amplifying pKD4 with oligonucleotides yaiWREDF and yaiwREDR. Confirmation of this mutant was performed via PCR with oligonucleotides yaiwEXTF and yaiwEXTR and compared with the product from the wild type strain.

The ability of Δ*yaiW* to grow in liquid culture at 37°C, 42°C and 48°C was assessed and demonstrates no difference in growth kinetics between itself and its parent strain. There was no significant difference between the sensitivities of SL1344 and Δ*yaiW* for all of

the agents tested against Δ*sbma* via disc diffusion assay, unlike Δ*sbmA* which was more sensitive to stress generated by the aminoglycoside antibiotic gentamicin and H_2O_2.

The only other significant phenotype discovered for the Δ*sbmA* mutant thus far is a reduced ability to colonise the spleen and liver of mice after intraperitoneal infection. We performed a similar intraperitoneal competition assay in BALB/c mice using a mixed dose of $5x10^3$ CFU/200µl dose of wild type SL1344 and the Δ*yaiW* mutant. Mice were culled on day 3 and log CFU/organ analysed for the spleens and livers. Figure 36 depicts this data, with a mean log CFU/organ of approximately $5x10^5$ for both the wild type strain and the Δ*yaiW* mutant isolated from both the spleens and liver. This equates to a CI of 1.0, with no significant difference between survival of Δ*yaiW* and the wild type parent strain in BALB/c mice via this route.

7.6 Discussion

sbmA became of particular interest to us after identification of its putative regulation by σ^E via both microarray analysis and a two plasmid screen, and from characterisation of a *Brucella* homologue where Δ*sbmA* has been proposed as a good vaccine candidate. Using a *sbmA* promoter reporter fusion containing a putative σ^E dependent promoter identified in our genome wide promoter search we have investigated the regulation of *sbmA* in *S.* Typhimurium. Under normal growth conditions the *sbmA* promoter is relatively inactive considering the entire predicted regulatory region of this gene has been fused with *lacZ*. This is consistent with the activity of this promoter in *E. coli* (Lavina, Pugsley, and Moreno, 1986). However a 2 fold increase in promoter activity does occur upon entry into stationary phase, which is consistent with a σ^E dependent promoter. Overexpression of σ^E under the control of arabinose, results in the dramatic

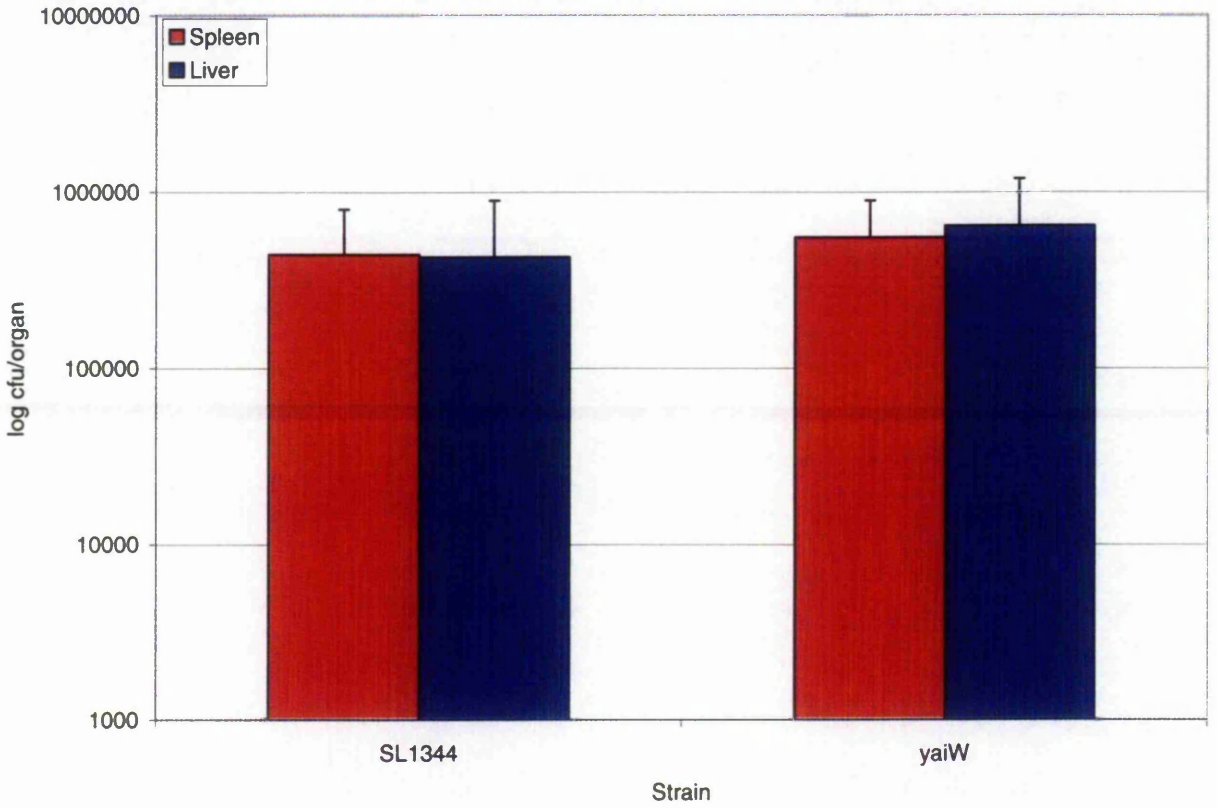


Figure 36. Intraperitoneal competition assay between Δ*yaiW* and the wild type *S.* Typhimurium parent strain.
A mixed dose of ~10^3 CFU/200µl was administered by the stated route. The log CFU/organ is a mean of 5 mice and the error bars represent standard deviation of the mean.

increase in *sbmA* promoter activity consistent with that seen with known σ^E dependent promoters of *rpoE* and *rpoH* in chapter 4 of this study, and confirms the results obtained from microarray analysis. Further indirect evidence of the presence of a σ^E dependent promoter was obtained by analysing the activity of the *sbmA* promoter fusion in a *surA* mutant background. We and others (Dartigalongue, Missiakas, and Raina, 2001) have used this genetically altered background to identify σ^E dependent promoters as a result of the build up of outer membrane proteins that occurs within the periplasm of this strain (Rouviere and Gross, 1996). Under these conditions the activity of the *sbmA* promoter is induced approximately 9 fold. Such an increase in *sbmA* promoter activity has been described in *S. meliloti* in an *htrA* mutant background (Glazebrook, Ichige, and Walker, 1996). This was explained as the reduced ability of a *htrA* mutant strain to degrade the chimeric alkaline phosphatase, although a similar induction was not seen with other promoter Tn*phoA* fusions. In light of our identification of *sbmA* being σ^E regulated in *S.* Typhimurium this phenomena might now be explained as *sbmA* playing a role in the ESR of *S. meliloti*. The marked increase in activity of the *sbmA* promoter in the absence of σ^E is indicative of another non-σ^E regulated promoter involved in the transcriptional regulation of *sbmA*. Such an increase has also been observed with the promoter regions of *fkpA* and *htrA* (GR, data not shown). There are several candidates for the other regulatory factor(s) of *sbmA* including CpxR, RpoS, RpoN and σ^H. The absence of RpoN and RpoS has no affect on *sbmA* promoter activity nor does inactivation or constitutive activation of the *cpxRA* two component system (GR, data not shown). The prime candidate must therefore be regulation by σ^H. We cannot measure the activity of the *sbmA* promoter in the absence of σ^H due to our inability to construct an *rpoH* mutant strain. However, we have already shown that the absence of functional σ^E leads to an induction of σ^H transcription (chapter 4). We have searched for

a putative σ^H regulated promoter upstream of *sbmA*, but cannot identify anything that corresponds with a promoter consensus for this alternative sigma factor (Ramirez-Santos *et al.*, 2001). Activity of the *sbmA* promoter in a Δ*sbmA* background is identical to that seen in the wild type strain and suggests no autoregulation mechanism.

Analysis of a *S.* Typhimurium Δ*sbmA* mutant was particularly revealing and another case of differential phenotypes of closely related genes of *S.* Typhimurium and *E. coli*. The *E. coli sbmA* mutant behaves similarly to that of *S. meliloti* in its increased resistance to gentamicin, bleomycin, microcins B17 and J25 and are indeed functionally equivalent to a certain extent. Loss of functional SbmA in *S.* Typhimurium does lead to an increased resistance to gentamicin but has no affect on bleomycin resistance. The natural resistance of *S.* Typhimurium to microcins has been attributed to a lack of functional FhuA, and when *E. coli fhuA* is incorporated into the *S.* Typhimurium chromosome, the resulting strain becomes sensitive to microcins. Further investigations of the role of SbmA in *S.* Typhimurium should include analysis of a *sbmA* mutant in the presence of FhuA to see if this corrects the differential phenotypes observed.

The *Brucella* species are close phylogenetic relatives of the *Rhizobia* (Moreno *et al.*, 1990) and within their separate intracellular environments are exposed to similar conditions such as reduced pH and reactive oxygen intermediates (Roop *et al.*, 2002). The inability of a *Sinorhizobium sbmA* mutant to form a long term symbiotic state with infected alfalfa plants (Long, McCune, and Walker, 1988) spurred research into the ability of a *Brucella abortus sbmA* mutant to survive within murine macrophages and within a murine model of infection. This mutant was more susceptible to phagocyte killing after 12 hours than the parent strain, but showed no phenotype *in vivo* until 2

weeks post infection, when the *sbmA* mutant was rapidly cleared (LeVier *et al.*, 2000). This strain still has the ability to illicit strong *Brucella* specific humoral and cellular responses, and provides protection against subsequent challenge (Roop *et al.*, 2002). Interestingly the loss of *sbmA* in *S.* Typhimurium does result in an increased sensitivity to both hydrogen peroxide and paraquat, again indicative of its involvement in the σ^E regulon. One might be tempted to presume that this increased susceptibility to oxidative stress might result in a loss of survival within murine macrophages, as observed in *Brucella.* However this does not appear to be the case with equal numbers of the wild type strain and the Δ*sbmA S.* Typhimurium strain isolated from murine macrophages after both 3 and 24 hours post infection. There is also no significant difference between the survival/replication of *S.* Typhimurium Δ*sbmA* and the wild type strain after IP infection of BALB/c mice. The *Brucella abortus sbmA* mutant is attenuated in a mouse model but the effects seen are after long term infection. To ascertain if SbmA plays a role in *S.* Typhimurium persistent infection, a model for persistence needs to be used, such as that described by (Clements *et al.*, 2002). However use of ity^R mice did allow us to measure CFU/organ up to 10 days post infection. At this later time point, the difference between CFU/organ of the wild type strain and the Δ*sbmA* mutant had increased with a CI of 0.2 in relation to a CI of 0.5 for the 3 day time point in both BALB/c and DBA/2 mice, suggestive of a role in persistence.

Seven days post oral infection of BALB/c mice with Δ*sbmA,* although numbers were again reduced with respect to the wild type strain, there were still greater than 10^6 CFU/organ in both the spleens and livers and mice had to be culled due to project licence restrictions. In combination with a *htrA* mutant (BRD915), there was no difference in CFU for any of the organs investigated after oral infection. Out of five

mice orally challenged with 10^{10} CFU of wild type SL1344 after a previous oral inoculation with 10^{7} of the Δ*htrA* Δ*sbmA* double mutant all five mice survived, as did the mice inoculated with the single *htrA* mutant.

Deletion of the downstream gene of *sbmA*, *yaiW*, has no affect on *S.* Typhimurium under any of the conditions we have investigated and possesses none of the limited phenotypes identified for the *sbmA* mutant. The protein sequence of YaiW is unrelated to anything else in the database.

At this stage the role of the *sbmAyaiW* operon in the ESR of *S.* Typhimurium still remains unclear although SbmA does appear to contribute to resistance to oxidative stress and gentamicin.

Chapter 8 - Construction and Characterisation of *S.* Typhimurium Δ*tolR* and Δ*skp* Mutants

8.1 Introduction

This chapter covers two putatively σ^E regulated genes, *skp* and *tolR* (identification discussed below). They have been grouped together in this chapter not through a functional relationship, but because although they both appear to possess interesting phenotypes, there has only been time to perform limited characterisation at this stage, and each lack the complementation studies required confirming these preliminary phenotypes. Each gene will be introduced and discussed individually.

8.1.1 Skp

Skp (seventeen kDa protein) is thought to be a periplasmic chaperone (Thome and Muller, 1991), although its role is still to be completely elucidated. Consistent with an extracytoplasmic location, the protein is synthesised as a precursor containing a signal sequence in the N terminal (Thome and Muller, 1991). The name Skp was coined by Kleppe and co-workers (Holck and Kleppe, 1988) who identified the *E. coli* gene, although the protein was first purified in 1979 as an LPS associated protein of *Salmonella minnesota* (Geyer *et al.*, 1979). Its initial function in *E. coli* was thought to be that of a histone like protein, resulting in one of the other names for this protein (HlpA) (Lathe *et al.*, 1980) and for some time was mistaken as the product of the downstream gene, *firA* (Aasland *et al.*, 1988). Skp was also purified by Chen and Henning (Chen and Henning, 1996) on the basis that it was the predominant protein bound to unfolded OmpF in an affinity chromatography assay. The *S.* Typhimurium homologue was identified in 1989 and assigned the third and final name for this gene, *ompH* (Koski *et al.*, 1989;Koski, Hirvas, and Vaara, 1990). An *E. coli skp* null mutation leads to a moderate reduction in proteins in the outer membrane (Chen and Henning, 1996), but confers to no determinable phenotype that indicates alterations to cell

viability of the organism (Rizzitello, Harper, and Silhavy, 2001). However the combination of a *skp* null mutant with a *surA* null mutant confers a lethal phenotype, but not when combined with a *htrA* null mutant (Rizzitello, Harper, and Silhavy, 2001). This same group proposes two pathways of chaperone activity in the periplasm based on this evidence, with HtrA and Skp in one pathway and SurA in the other and predict that Skp and HtrA share a redundant function with SurA, the function being periplasmic chaperone activity. Lethality of a Δ*skp* Δ*fkpA* double mutant has also been described (Dartigalongue, Missiakas, and Raina, 2001), which puts FkpA in the same pathway as SurA in the proposed model.

The biochemical role of Skp as a periplasmic chaperone is starting to be unravelled. Data now available suggests that Skp participates in the early folding events of outer membrane proteins such as OmpA (de Cock *et al.*, 1999). Skp binds an NH_2 terminal region of OmpA immediately after folding begins (Schafer, Beck, and Muller, 1999). The crystal structure of Skp has now been solved by two groups (Korndorfer, Dommel, and Skerra, 2004;Walton and Sousa, 2004). It forms a trimer both in solution (Schlapschy *et al.*, 2004) and in the protein crystal, with an overall basket-like shape. A comparison of the relative sizes of OmpA and Skp, suggests that OmpA is bound in a compacted state, as the unfolded state would be too large to be accommodated by Skp and would therefore be unprotected from interacting with other polypeptides (Korndorfer, Dommel, and Skerra, 2004). Skp is also firmly bound to the plasma membrane via interactions with phospholipids and like HtrA cannot be released from the cell following osmotic shock (Pallen and Wren, 1997). Most interestingly a Δ*skp*::Tn10 mutant was shown to induce the σ^E regulon in *E. coli*, indicative of an increase in misfolded outer membrane proteins within the periplasm (Missiakas, Betton, and Raina, 1996). In chapter 4 of this thesis we have also demonstrated that the

autoregulated *rpoEP3* promoter of *S.* Typhimurium is also induced by a mutation in *skp*. In chapter 5 we have also discussed that unlike in *E. coli* (Dartigalongue, Missiakas, and Raina, 2001) we cannot thus far identify a promoter region upstream of *skp* consistent with σ^E regulation.

Recently, *E. coli* Skp has been identified as a chemoattractant of monocytes and polymorphonuclear leukocytes via the C5a receptor, one of the most important leukocyte receptors involved in the inflammatory reaction. As Skp is widely distributed in Gram negative bacteria the recognition of Skp by leukocytes could be of benefit to the host innate immunity against Gram negative bacteria (Shrestha *et al.*, 2004).

8.1.2 TolR

In *E. coli* the *tol* genes are transcribed from two promoters resulting in two transcripts one consisting of *ybgC tolQRA* and one consisting of *tolB pal ybgF* (Vianney *et al.*, 1996). *E. coli* TolQRA is the basic system for import of all group A colicins and filamentous phage DNA (Webster, 1991). There is a degree of homology between the *E. coli* TolQRA and ExbBDTonB systems at both the DNA and amino acid levels (Eick-Helmerich and Braun, 1989). TolQ and TolR also share a degree of functional equivalence with ExbB and ExbD respectively, as exchange of these proteins in genetically altered backgrounds results in restoration of the specific uptake pathway of colicins (Braun and Herrmann, 1993). Colicins are bacterial toxins which are active against *E. coli* and related bacteria. These folded proteins cross the membrane barriers by recognising an outer membrane receptor and then interact with proteins in the periplasmic space to reach their target. Group A colicins use the Tol system for translocation, the most studied of which bind the BtuB outer membrane protein (high affinity transporter of vitamin B_{12})(Lazzaroni, Dubuisson, and Vianney, 2002).

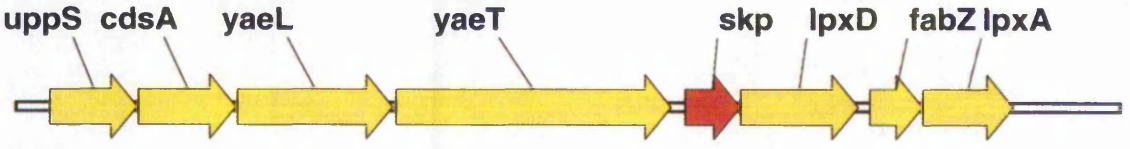
E. coli Tol proteins seem to be specifically involved in the maintenance of an intact outer membrane with mutations in these genes resulting in leakage of the periplasmic constituents (Fognini-Lefebvre, Lazzaroni, and Portalier, 1987;Lazzaroni, Fognini-Lefebvre, and Portalier, 1989). Tol mutants are also hypersensitive to agents such as detergents, quaternary compounds and aminoglycosides and induce capsule synthesis resulting in a mucoid phenotype (Vianney *et al.*, 1994).

A model for the Tol complex has been proposed, in which it completely spans the cell envelope (Bouveret *et al.*, 1995). This same group propose that any disruption of this complex would result in a destabilisation of the physical attachment of the peptidoglycan and the link between the inner and outer membranes. Vianney *et al.*, 1994 have shown that TolQ is an inner membrane protein with three transmembrane domains and two cytoplasmic regions. TolR and TolA have a three domain structure consisting of N terminal, central and C terminal domains. The C terminal forms an amphiphatic helix which interacts with the cytoplasmic membrane and is anchored to the cytoplasmic membrane by a single membrane spanning segment near the N terminus resulting in most of the protein being exposed to the periplasm (Levengood, Beyer, Jr., and Webster, 1991;Muller *et al.*, 1993;Journet *et al.*, 1999). The transmembrane fragment of TolR and the third transmembrane domain of TolQ are involved in the proton motive force (pmf) dependent activation of TolA (Cascales *et al.*, 2000). TolQ and TolR are also homologous to MotA and MotB components of the flagella motor, it is hypothesised that the transmembrane domains of TolQRA constitute an ion potential driven motor (Cascales, Lloubes, and Sturgis, 2001).

The exact role of the *tol-pal* operon (section 8.5) remains to be elucidated but the high degree of conservation of this system and the fact that some of the *tol-pal* genes are required for cell viability in *P. aeruginosa* (Dennis, Lafontaine, and Sokol, 1996), *E. coli* 0157 (Gaspar *et al.*, 2000) and *Haemophilus ducreyi* (Spinola *et al.*, 1996) reflects an important role for the *tol* system. In *P. aeruginosa*, expression from *tolR* is dependent on the *tolQ* promoter, with *tolQR* transcribed as an operon, and *tolA* transcribed on its own. *P. aeruginosa tolQ* or *tolR* genes did not complement the respective mutants in *E. coli.* Interestingly inactivation of *tolQ* or *tolA* is lethal in *P. aeruginosa* indicative of different roles for these genes and their products in comparison with *E. coli* K12 (Dennis, Lafontaine, and Sokol, 1996).

In *S.* Typhimurium little investigation has been completed on the *tol-pal* system. However in a screen for genetic loci involved in the resistance of *S.* Typhimurium to bile, three of the unique MudJ inserts identified were closely linked to *tol* or *tol* associated genes (Prouty, Van Velkinburgh, and Gunn, 2002). This same group also show that RcsCB does not regulate transcription of the *tol* genes in *S.* Typhimurium as it does in *E. coli* (Clavel *et al.*, 1996). Other differences between the *tol* mutants of *S.* Typhimurium and *E. coli* are that *S.* Typhimurium mutants have no growth defect with respect to the wild type strain and have no mucoid phenotype.

8.2 Genetic Orientation of *skp*



skp is located at 4 minutes on the *S.* Typhimurium genetic map. The genes upstream of *skp* have been identified as members of the σ^E regulon in *E. coli*, with *yaeL* essential both for activation of the σ^E pathway and cell viability. In *S.* Typhimurium we have

identified an σ^E regulated promoter located within the CDS of *yaeL* and putatively regulating transcription of *yaeT*, but thus far no promoter has been identified upstream of *yaeL* in *S.* Typhimurium. The downstream gene to *skp*, *lpxD*, encodes for an acyltransferase involved in formation of lipid A biosynthesis, therefore polar mutants would almost certainly be lethal. To ensure that the *skp* mutant phenotypes described in this chapter are a direct result of this mutation and are not a result of a polar effect on *lpxD*, complementation studies need to be completed. However, these studies are not always capable of complementing *in vivo* phenotypes (Halsey *et al.*, 2004).

8.3 Construction of a Δ*skp S.* Typhimurium Mutant and *in vitro* Analysis

Using λ red mutagenesis with oligonucleotides skpRedF and skpRedR, the *S.* Typhimurium strain 12023 *skp* CDS was replaced with a kanamycin cassette from pKD4. Mutagenesis was confirmed via PCR with primers skpEXTF and skpEXTR, which in a wild type strain background should produce a 1Kb product. A comparison of the wild type and mutant products are depicted in figure 37. After mutagenesis the mutation was moved by P22 transduction to a SL1344 background strain (GVB1367) on which all the remaining characterisation was performed. We were also interested in trying to make a double mutation that had been described as lethal in *E. coli* (*fkpA*/*skp*) and a double mutant that had been described as viable in *E. coli* (*htrA*/*skp*). The Δ*htrA* Δ*skp*::kan double mutant was constructed by P22 transduction of the Δ*skp*::kan mutation into a BRD915 (Δ*htrA*) background, resulting in strain GVB1375. The *skp* and *fkpA* mutants were both marked with a cassette encoding for kanamycin resistance. To make a double mutant the kanamycin cassette was removed from the Δ*skp*::kan mutant using plasmid pCP20 (described in materials and methods) to leave an unmarked Δ*skp* mutant. The *fkpA*::kan mutation was then introduced into this strain by P22 transduction

to create strain GVB2021. The double mutants were again confirmed by PCR analysis of each individual mutation within the double, and products compared to those from the single mutants from which they were created and the wild type (GR, data not shown). Unlike in *E. coli* a *S.* Typhimurium Δ*skp fkpA::kan* double mutant is viable at 37°C, as is the Δ*htrA Δskp::kan* combination.

As with *E. coli* there is no evidence of loss of viability of the single *S.* Typhimurium *skp* mutation when grown at 37°C in liquid media (figure 38), indeed the double mutants are also equally as healthy under these conditions. The strains also grow equally as well as each other at 42°C (GR, data not shown), however, at 46°C the *skp* mutant demonstrates a marked growth defect in comparison to the wild type strain (figure 39). The BRD915 (Δ*htrA*) and GVB1375 (Δ*htrA*/Δ*skp)* double mutant grow equally as poorly as each other at 46°C, as is expected due to the temperature sensitive phenotype of an *htrA* mutant in our hands. Confusingly the *fkpA/skp* double mutant repeatedly grows better than the single *skp* mutant (GR, data not shown). This data is from the Bioscreen C machine and curves will need to be repeated in 50ml flask cultures for confirmation of results and before conclusions can be drawn, to ensure that this result is not an artefact of this method (i.e nutrient or aeration limitation).

The *skp* mutant demonstrates a significantly ($P<0.05$, student t test) increased sensitivity to the antimicrobial peptide polymixin B (100U) (Figure 40). No difference between the sensitivity of the mutant and the wild type strain for 3% hydrogen peroxide was observed.

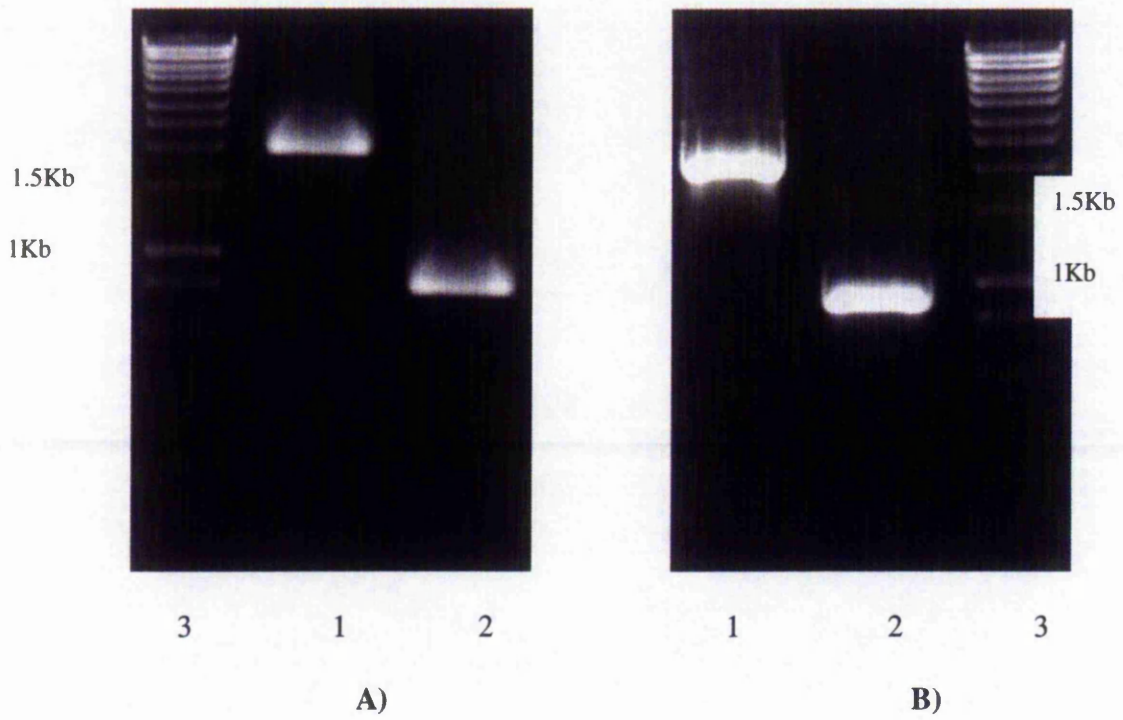


Figure 37. Confirmation of A) *skp* and B) *tolR S.* Typhimurium mutants by PCR analysis.

Each 1% TBE electrophoresis gel picture contains mutant (1) and wild type (2) bands along with a DNA ladder (3). 20µl of a 50µl PCR reaction is loaded for wild type and mutant, with 5µl of Hyperladder I (Bioline). The respective sizes and primers used are described in the text.

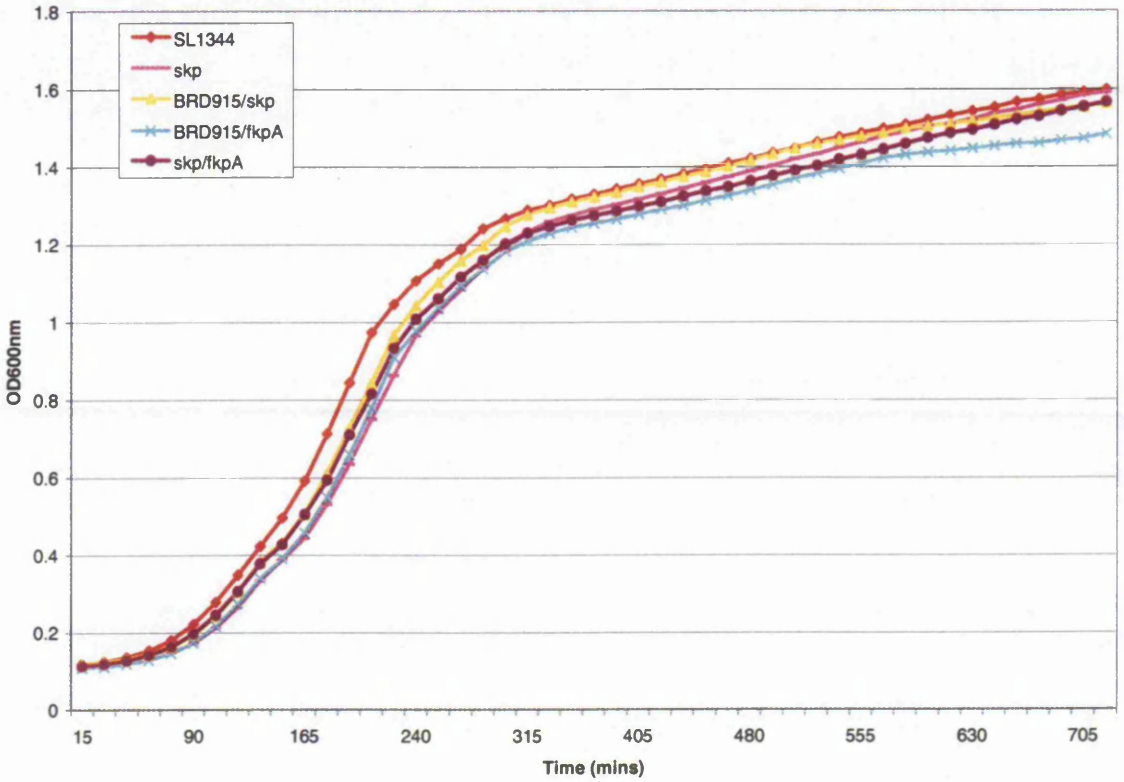


Figure 38. Growth Kinetics of *S.* Typhimurium Δ*skp* single and double mutants at 37°C.

Data presented here is a mean of triplicate data per strain, obtained from growth at 37°C with shaking on the Bioscreen C machine. The OD600nm was measured and recorded every 15 minutes. Dilutions and aliquot size used are detailed in the Materials and Methods chapter.

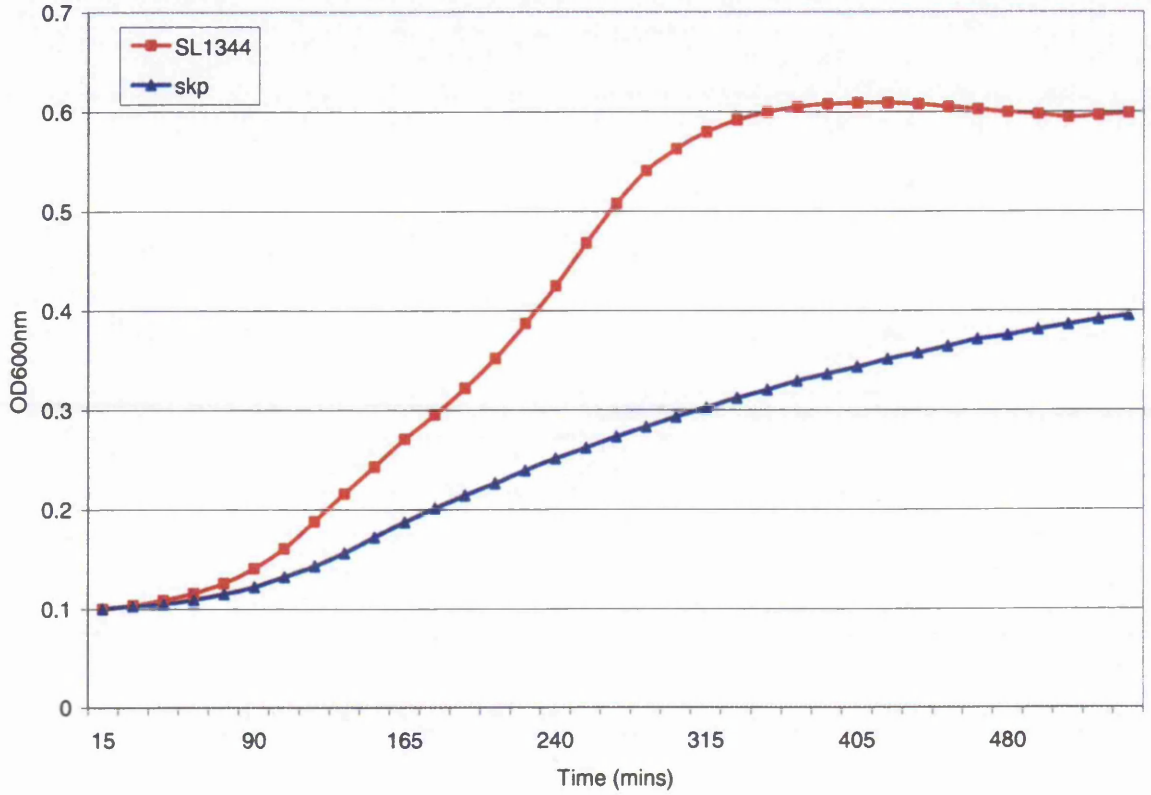


Figure 39. Growth kinetics of a *S.* Typhimurium Δ*skp* mutant in liquid media at 46°C.
Data presented here is a mean of triplicate data per strain, obtained from growth at 46°C with shaking on the Bioscreen C machine. The OD600nm was measured and recorded every 15 minutes. Dilutions and aliquot size used are detailed in the Materials and Methods chapter.

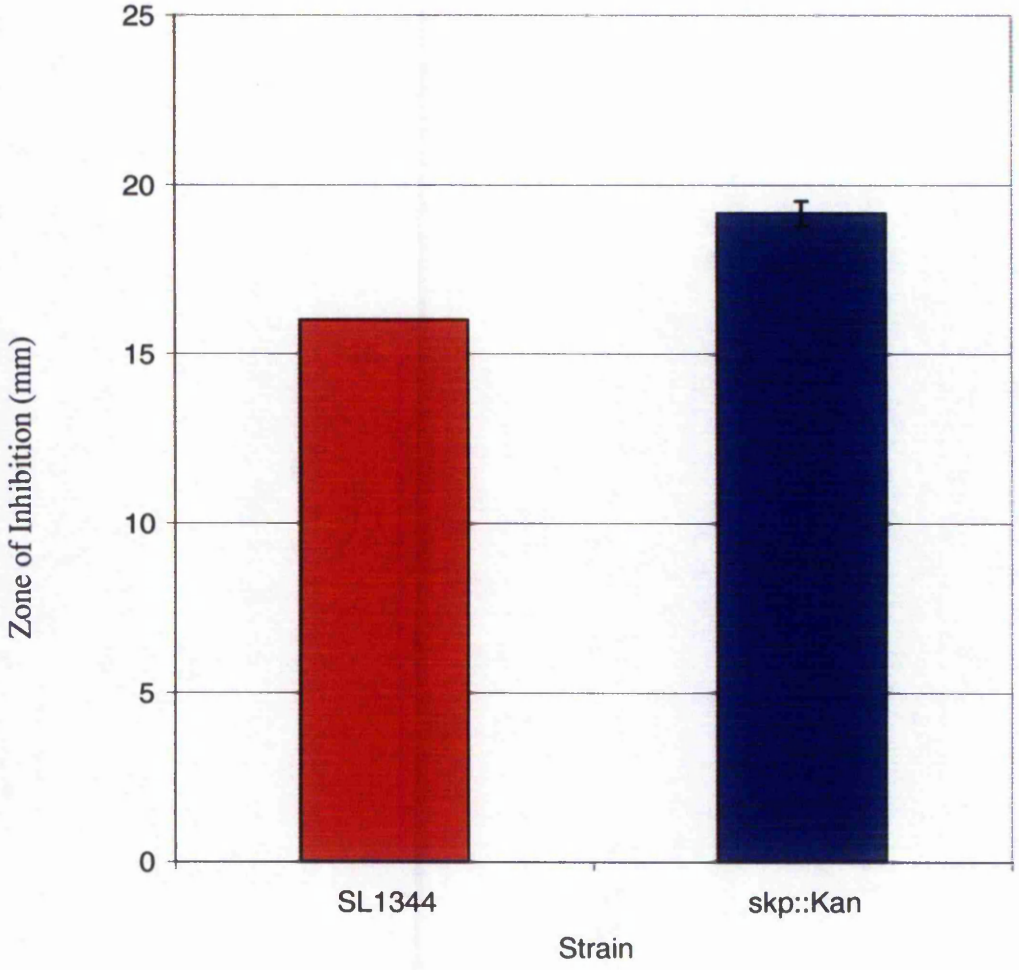


Figure 40. Sensitivity of a *S.* Typhimurium *Δskp* mutant to the antimicrobial peptide, polymixin B.

Using a disc diffusion assay (Materials and Methods), sensitivity of wild type *S.* Typhimurium and a *Δskp* mutant to 100U polymixin B was compared. Data presented here is a mean of 30 data points and the error bars represent the standard deviation from the mean.

8.4 Analysis of Δ*skp* in a Murine Model of Infection.

Initially a competition assay was performed using a mixed dose containing ~$2x10^3$ CFU of both the wild type strain and the Δ*skp*::kan mutant per 200µl. The dose was administered via the IP route to 5 female BALB/c mice. After 3 days, spleens and livers from the infected mice were harvested and viable counts of each strain calculated. Figure 41 depicts the average log CFU/organ isolated from both spleens and livers. Less than 10^4 CFU/organ of the *skp* mutant could be detected in either organ at this time point, in comparison to 10^5 of the wild type strain. After correcting for slight differences in dose the CI of *skp* is 0.04 and is significantly different to the wild type strain ($p<0.05$, student t test).

As a *skp/htrA* double mutant is viable we were intrigued to see if the addition of the *skp* mutation further attenuated the already attenuated *htrA* (BRD915) strain. A mixed dose containing $1x10^3$ of both BRD915 and BRD915 Δ*skp*::kan strains was administered to 3 BALB/c mice in a 200µl dose via the IP route. After 3 days a slight reduction in the CFU/spleen was observed for the double mutant ($9x10^2$) in comparison with BRD915 ($6x10^3$), although the CFU/liver were almost identical for both strains (figure 42) Overall, when all data points are taken into account there is no significant difference between BRD915 and BRD915 Δ*skp*::kan after systemic infection.

Single oral infections were used to investigate the effects of a *skp* mutation on *S.* Typhimurium infection via the natural route of infection (figure 43). $7.4x10^6$ CFU/200ul dose was administered by oral gavage to 5 BALB/c mice and compared to a group of 5 mice dosed with $5x10^5$ of wild type SL1344. An average CFU/organ was calculated five days post infection for the spleen, liver, MLN and Peyer's patches. For all organs other

than the Peyer's patches the CFU/organ of the *skp* mutant was some two logs less than for the wild type strain. In the Peyer's patches however, the numbers of Δ*skp* isolated were a log fold greater than the wild type strain, which is as expected due to the higher dose of this strain administered in comparison to the wild type strain. The CFU/organ for each organ investigated, other than the Peyer's patches is significantly reduced (student t test, $p<0.05$) in the *skp* mutant when compared with the wild type strain, indicating a defect in deeper tissue survival.

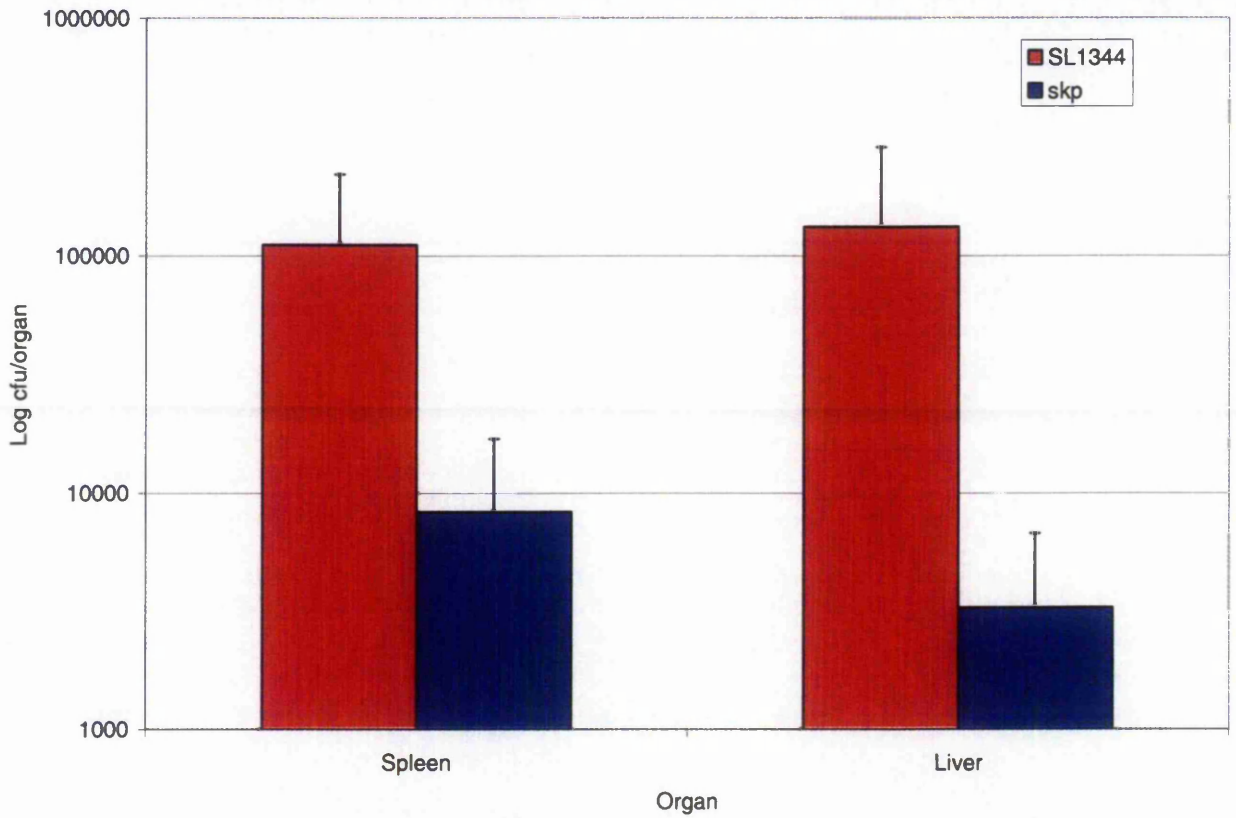


Figure 41. Competition Assay of wild type *S.* Typhimurium v Δ*skp* in BALB/c mice via the IP route of infection.

A group of 5 BALB/c mice were infected with a 200µl mixed dose containing ~$2x10^3$ of both wild type and Δ*skp S.* Typhimurium. Mice were culled on day 3 and spleens and livers harvested for viable counts of each strain per organ. The data presented here is a mean of the 5 mice (corrected for dose) and the error bars represent the standard deviation from the mean.

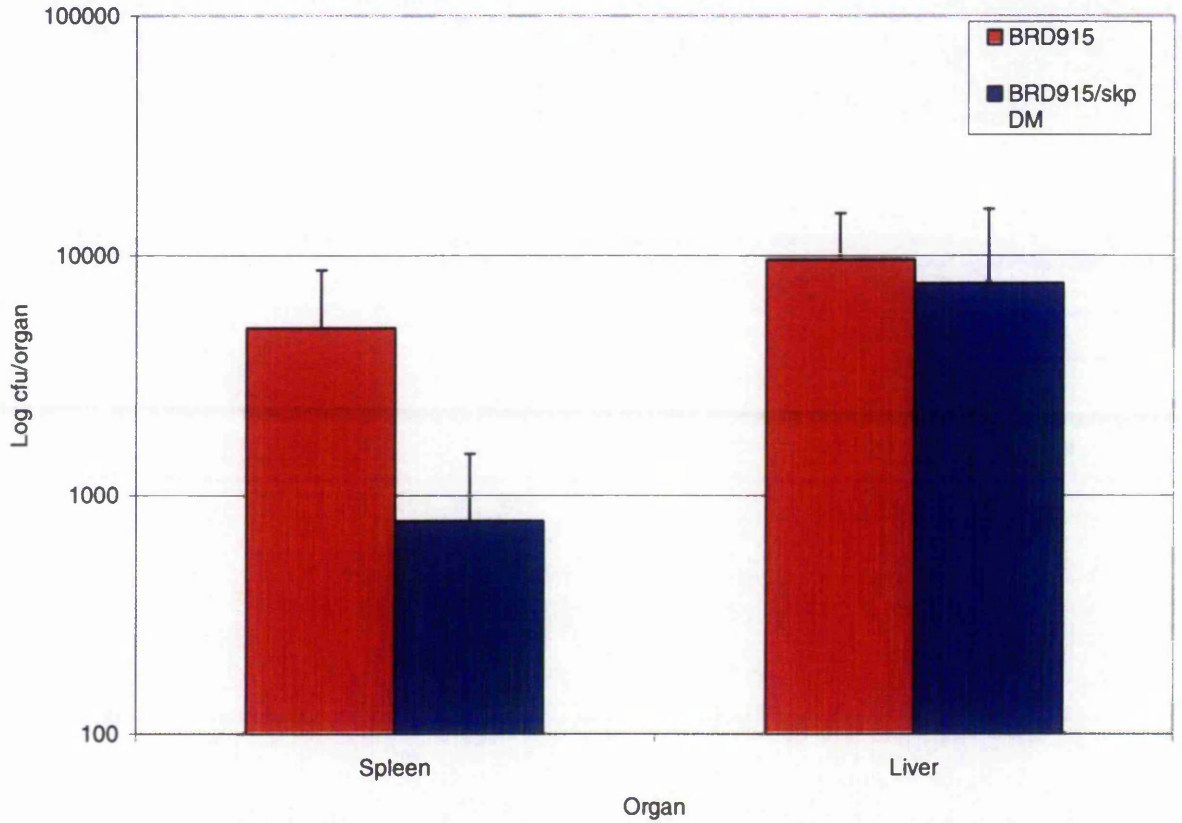


Figure 42. Competition Assay of BRD915 v BRD915Δ*skp* in BALB/c mice via the IP route of infection.

A group of 3 BALB/c mice were infected with a 200µl mixed dose containing ~$1x10^3$ of both BRD915 and BRD915Δ*skp S.* Typhimurium. Mice were culled on day 3 and spleens and livers harvested for viable counts of each strain per organ. The data presented here is a mean of the 3 mice (corrected for dose) and the error bars represent the standard deviation from the mean.

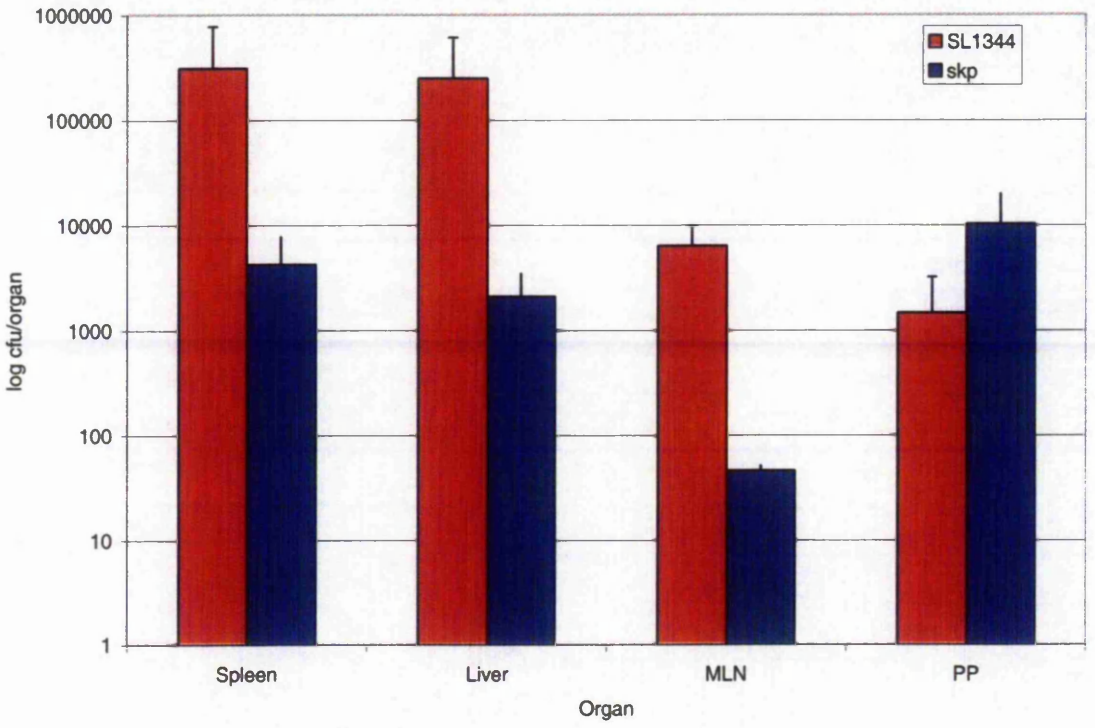
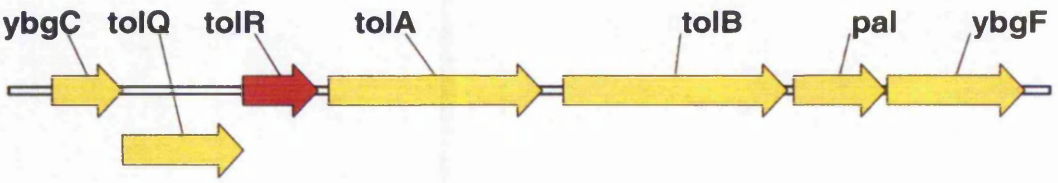


Figure 43. Oral Infection of BALB/c mice with wild type and Δ*skp* *S.* Typhimurium.

Mice were inoculated orally with either $5x10^5$ CFU of the WT strain or $7.4 x10^6$ CFU of the *skp* mutant and the number of bacteria present in different organs determined 5d later. The bar represents the mean of 5 mice and the error bar indicates the SD. PP, Peyer's patches: MLN, mesenteric lymph node.

8.5 Genetic Orientation of *S.* Typhimurium *tolR*



tolR is located between base pairs 814214 and 814552 on the *S.* Typhimurium genetic map. From characterisation in *E. coli* it is thought that *tolR* is part of an operon with *ybgC*, *tolQ* and *tolA*. A disruption in *tolR* could therefore lead to a disruption of *tolA*. However, if our aim is to investigate the role of the Tol pathway in *S.* Typhimurium virulence then this is not a problem, but in order to assign any particular phenotype directly to *tolR*, complementation will need to be performed. The Kormanec laboratory by S1 mapping and ourselves by consensus search have identified a putative σ^E dependent promoter within the CDS of *tolQ* (see chapter 5), indicating that in *S.* Typhimurium *tolQ* and *tolR* may not be genetically coupled.

8.6 *in vitro* Analysis of a *S.* Typhimurium Δ*tolR*::kan Mutant.

Our SL1344 Δ*tolR*::kan (GVB1360) mutant was constructed by red mutagenesis using primers tolRREDF and tolRREDR, resulting in complete deletion of the entire CDS of *tolR* and insertion of a kanamcyin cassette from plasmid pKD4. Analysis was confirmed using primers tolREXTF and tolREXTR resulting in ~1 Kb product in wild type and ~1.5Kb product in a mutant (figure 37). In *E. coli tol* mutants have a slower growth rate than the parent strain at 37°C, and exhibit a mucoid colony phenotype (Bernstein, Rolfe, and Onodera, 1972;Vianney *et al.*, 1994). A *mudJ* insertion into the *tol* operon of *S.* Typhimurium demonstrates no altered growth at 37°C or has no colony phenotype

(Prouty, Van Velkinburgh, and Gunn, 2002). After analysis of our Δ*tolR* mutant we see a slight defect in growth at 37°C which exhibits a slightly protracted lag phase and enters stationary phase slightly earlier than the wild type strain (figure 44). No temperature sensitivity was observed with this mutant (GR-data not shown). The *S.* Typhimurium Δ*tolR* does not have a mucoid colony phenotype and does not demonstrate increased sensitivity to polymixin B, hydrogen peroxide or to an array of antibiotics tested.

8.7 Analysis of Δ*tolR* in a Murine Model of Infection

The Δ*tolR* mutant is extremely attenuated after administration by both systemic (IP) and natural routes. As mentioned above the *tolR* mutant could be defective in the entire *tolQRA* operon as we have not performed complementation. However we are the first group to show that the Tol pathway plays an important role in the pathogenesis of *S.* Typhimurium in a murine host.

After competition assay of ~10^3 CFU/dose of both the wild type strain and Δ*tolR* by the IP route of infection, the CFU/organ of each strain was ascertained for both spleens and livers on day 3 (figure 45). After calculating the mean of the three BALB/c mice infected, the CFU/organ for Δ*tolR* ($5x10^3$) was some 2.5 logs lower than the wild type strain ($1x10^6$) in both the spleen and liver. This equates to a CI of ~ 0.007 which is extremely significant ($p<0.01$, student t test) when compared with wild type *S.* Typhimurium.

As with all of the mutants that have demonstrated a significant level of attenuation after IP administration, we investigated the effects of a Δ*tolR* mutant on survival after oral infection. $6x10^6$ CFU/200μl dose was administered by oral gavage to 5 BALB/c mice and again organs were harvested five days post infection. Figure 46 shows the average

CFU/organ for each organ analysed. A four log 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 log 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 reduction seen in the Peyer's patches (8×10^1 versus 1×10^3). The CFU/organ for each organ investigated is significantly reduced (student t test, $p<0.05$) for the *tolR* mutant when compared with the wild type strain.

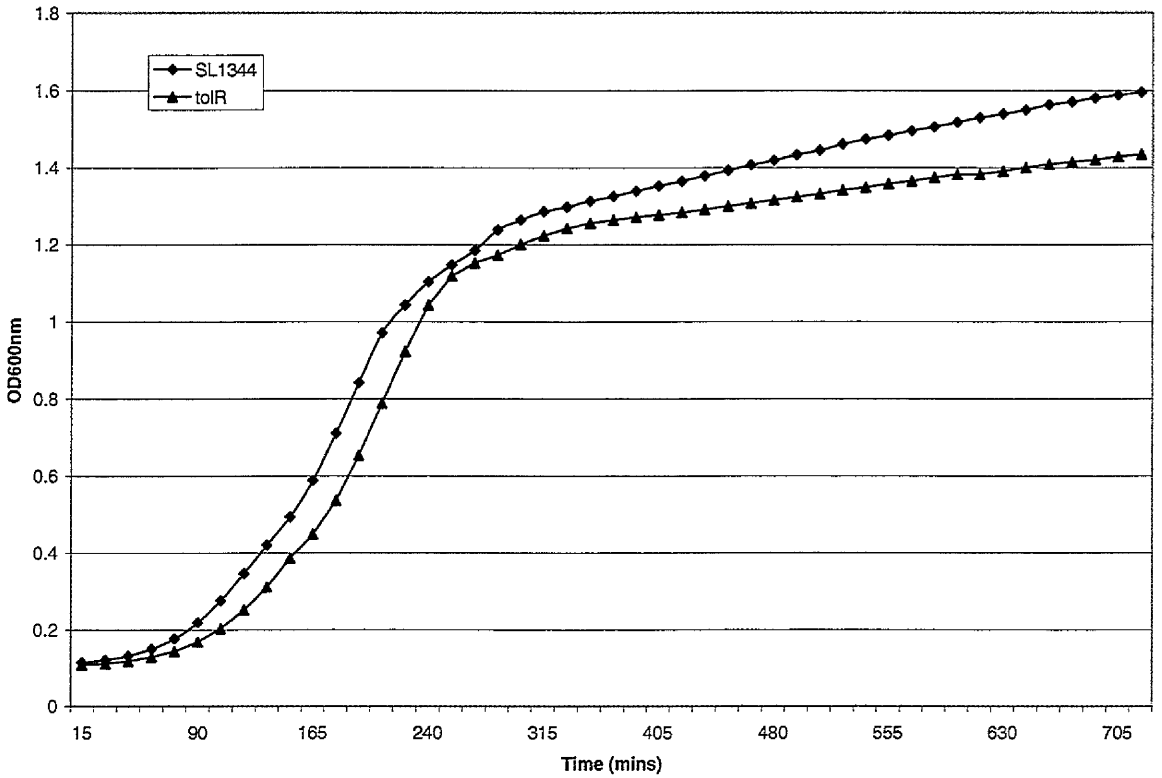


Figure 44. Growth kinetics of a *ΔtolR S.* Typhimurium mutant at 37°C.

Data presented here is a mean of triplicate data per strain, obtained from growth at 37°C with shaking on the Bioscreen C machine. The OD600nm was measured and recorded every 15 minutes. Dilutions and aliquot size used are detailed in the Materials and Methods chapter.

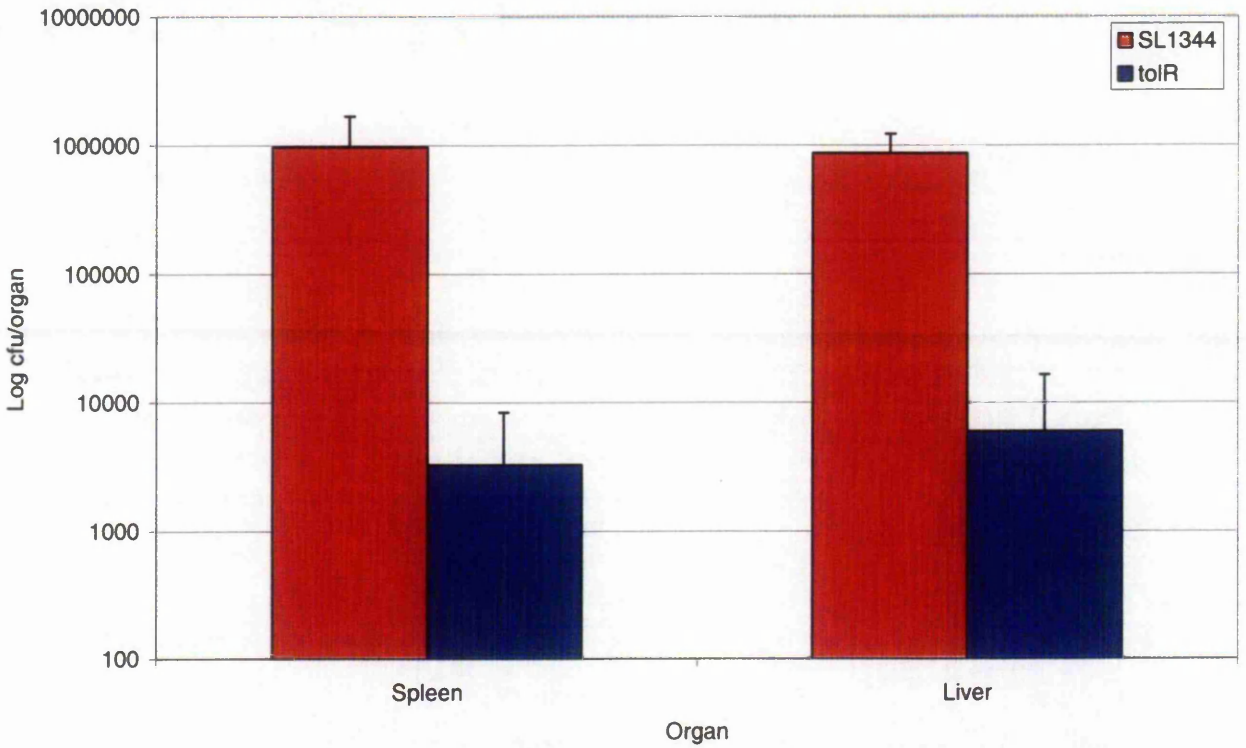


Figure 45. Competition assay of wild type *S.* Typhimurium v *ΔtolR* via the IP route of infection.

A group of 5 BALB/c mice were infected with a 200µl mixed dose containing ~$2x10^3$ of both wild type and *ΔtolR S.* Typhimurium. Mice were culled on day 3 and spleens and livers harvested for viable counts of each strain per organ. The data presented here is a mean of the 5 mice (corrected for dose) and the error bars represent the standard deviation from the mean.

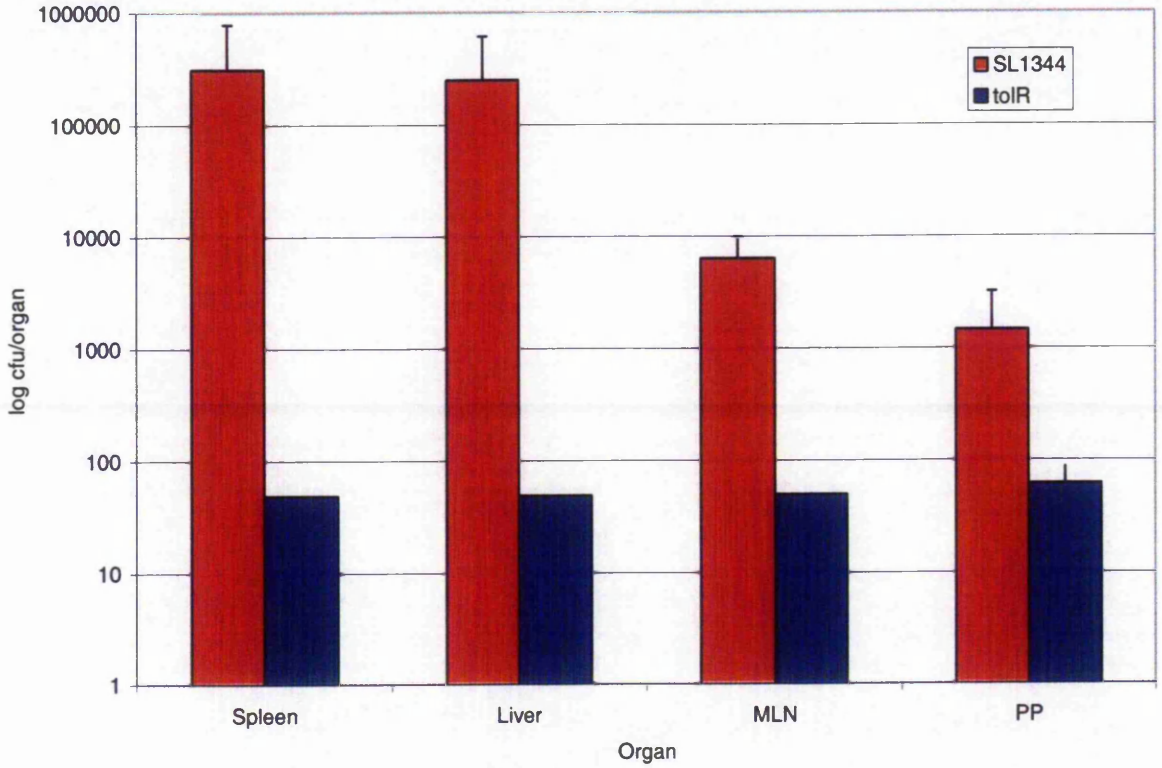


Figure 46. Comparison of oral infection of BALB/c mice with wild type *S.* Typhimurium and a *ΔtolR* mutant.

Mice were inoculated orally with either $5x10^5$ CFU of the WT strain or $6 x10^6$ CFU of the *tolR* mutant and the number of bacteria present in different organs determined 5d later. The bar represents the mean of 5 mice and the error bar indicates the SD. PP, Peyer's patches: MLN, mesenteric lymph node.

8.8 Discussion

The genes discussed in this chapter have been ascribed diverse functions but neither had been previously associated with pathogenesis of *S.* Typhimurium. We have demonstrated in this chapter that both are attenuated after systemic and oral infection of a murine model, but again need to stress that complementation studies which are currently being carried out, must be used to validate this data.

Regulation of *skp* still remains something of a quandary. Skp has been described in *E. coli* as σ^E regulated, and a putative promoter assigned (Dartigalongue, Missiakas, and Raina, 2001). However this promoter does not look anything like the promoter consensus we have derived from σ^E regulated promoters in *S.* Typhimurium. The confirmation of this promoter as being σ^E regulated is based on the increase in promoter activity in the absence of *surA* (Dartigalongue, Missiakas, and Raina, 2001). The promoter identified in *E. coli* is not located within 1000bp upstream of the gene in *S.* Typhimurium. From the data that we have available to us at the moment no σ^E dependent promoter can be identified in *S.* Typhimurium, either by promoter consensus search or S1 mapping. However, expression of this gene is greater than 7 fold increased after the over expression of σ^E, implicating a possible indirect role for σ^E regulation of *skp* and could explain the increase of the putative σ^E dependent *skp* promoter in *E. coli* when assayed in a *surA* mutant background.

What is certain is that loss of functional Skp within the bacterial cell of both *E. coli* and *S.* Typhimurium does result in an environment that leads to induction of the σ^E regulon, (Missiakas, Betton, and Raina, 1996) and this thesis. Phenotypes of the *S. Typhimurium skp* mutant are also consistent with an involvement in the σ^E regulon, particularly

temperature sensitivity at 46°C, sensitivity to polymixin B, and attenuation in a murine model. The level of attenuation of the *skp* mutant, although significant in comparison to the wild type strain and a number of other mutants tested in this thesis, does not possess the degree of attenuation of the *S.* Typhimurium *rpoE* mutant. It is however a candidate for immunological studies as a potential carrier or vaccine strain, as it is present in equal if not greater numbers than the wild type strain within the Peyer's patches, due to the higher dose of Δ*skp* administered, so one would imagine it is capable of eliciting a mucosal response. Whether the degree of attenuation seen is due to Skp being a chemoattractant (Shrestha *et al.*, 2004) should be investigated further. What is becoming clear from our characterisation of the σ^E regulon of *S.* Typhimurium is that maintenance of periplasmic homeostasis appears to be very important if the given strain is to survive within a murine host.

Differences between the σ^E regulons of *E. coli* and *S.* Typhimurium are detailed throughout this thesis and will be discussed further in the general discussion. Whether regulation of *skp* is another of these differences is still to be confirmed but the effect of combining mutations in *skp* with other known σ^E regulated genes is definitely another of these incidences. In *E. coli* mutants harbouring disruptions in both *skp* and *htrA* have been described (Rizzitello, Harper, and Silhavy, 2001). However a combination of *skp* and *fkpA* mutations has been described as a lethal combination at elevated temperatures (Dartigalongue, Missiakas, and Raina, 2001). In this chapter we have demonstrated that both Δ*skp*Δ*htrA* and Δ*skp*Δ*fkpA* double mutants are viable in *S.* Typhimurium at 37°C. In *E. coli* two pathways of chaperone activity in the periplasm have been described, with HtrA and Skp in one pathway and SurA in the other (Rizzitello, Harper, and Silhavy, 2001). They predict that Skp and HtrA share a redundant function with SurA,

the function being periplasmic chaperone activity. The level of attenuation of a Δ*skp*Δ*htrA* mutant is no greater than that of a single *htrA* mutant after IP inoculation, and this would also indicate that they are involved in the same pathway. It will be interesting to see whether a Δ*skp*Δ*fkpA* double mutant is more attenuated than a single *skp* mutation. A *S.* Typhimurium *fkpA* mutant is not particularly attenuated *in vivo* (Humphreys *et al.*, 2003), but this could be due to the overlapping functions of HtrA and Skp for this protein.

The Tol export system has previously been disrupted in *S.* Typhimurium (Prouty, Van Velkinburgh, and Gunn, 2002) but not in any other pathogenic bacteria. The role that this system plays in virulence has never been investigated, although a plethora of data has been published for this system it mainly centres upon its role in translocating group A colicins (Lazzaroni, Dubuisson, and Vianney, 2002). We have shown here that disruption of this system by deletion of *tolR* severely attenuates *S.* Typhimurium in the mouse after administration by both the systemic and natural routes, although no other phenotypes have been identified from the assays completed. There has been somewhat of a debate over the operon structure of this gene cluster, and the agreement thus far has been that *tolQRA* are co-transcribed (Vianney et al., 1996). However we have identified a putatively σ^E regulated promoter in *S.* Typhimurium within the CDS of *tolQ* which could regulate transcription of *tolR* or *tolRA*. This promoter has been verified by S1 mapping by the Kormanec group and it is only active in the presence of σ^E suggesting that regulation of this operon is more complex than originally thought.

An increased sensitivity to bile has previously been assigned to *mudJ* insertions in the *tolQRA* operon of *S.* Typhimurium (Prouty, Van Velkinburgh, and Gunn, 2002). Whether this increased sensitivity plays a role in the level of attenuation seen remains to

be validated. What has become apparent from this work is that the Tol Pathway should definitely been investigated further as a potential vaccine/drug target.

The genes described in this chapter are the epitome of what we hoped to discover from genes that are σ^E regulated. Further investigation into the role that such genes play in the virulence process will begin to explain the degree of attenuation seen with the *rpoE* mutant, and aid in a search for future vaccine targets.

Chapter 9 – *stm1250* and *stm1251* (*agsA*). The First *Salmonella* Specific RpoE Regulated Genes

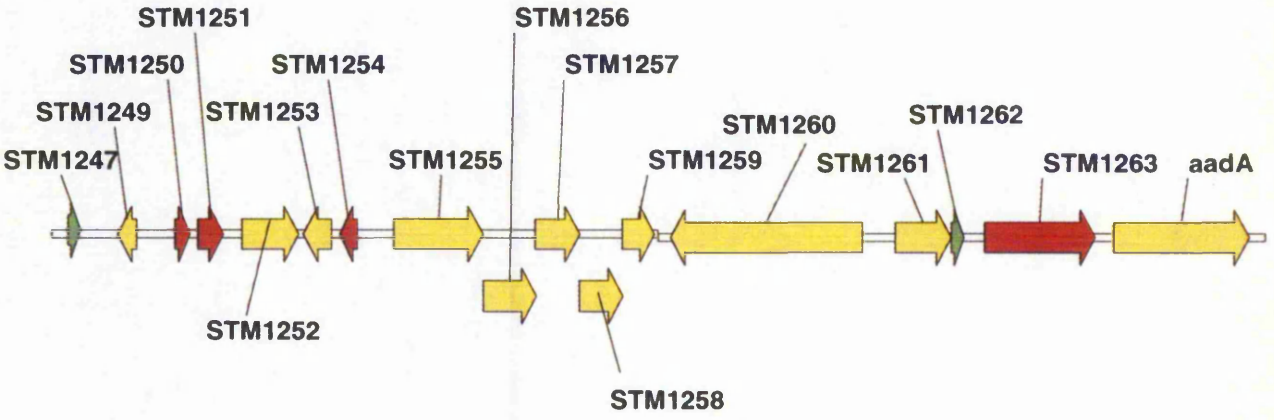
9.1 Introduction

This chapter covers the preliminary characterisation of four putatively σ^E regulated genes, *stm1250*, *stm1251* (*agsA*), *stm1254* and *stm1263*, and have been grouped together based upon their genomic location.

According to the *S.* Typhimurium LT2 genomic annotation (McClelland *et al.*, 2001) *stm1250* is a putative cytoplasmic protein, which from genomes sequenced thus far appears to be *Salmonella* specific. Stm1251 belongs to the Hsp20 family of small heat shock proteins which contain the same characteristic alpha-crystallin domain. These proteins are generally ATP-independent chaperones that prevent protein aggregation, and are important in refolding in combination with other Hsps (Bateman *et al.*, 2004). The closest relative to Stm1251 appears to be IbpA, which itself is only 33% parolagous by blastP (Altschul *et al.*, 1997). IbpA and IbpB prevent enzymes from inactivation by heat and oxidants (Kitagawa *et al.*, 2002), and also prevent the aggregation of endogenous denatured proteins (Kuczynska-Wisnik *et al.*, 2002). *stm1251* (*agsA*) was recently described by (Tomoyasu *et al.*, 2003) to be a novel heat shock gene encoding for a small chaperone, which when over-expressed, partially complements the thermo-sensitivity of a Δ*dnaK* mutant, and reduces the amount of heat-aggregated protein in both Δ*dnaK* and Δ*rpoH E. coli* mutants. This same group predict regulation of this gene by two promoters, one dependent on σ^{70} and the other on σ^{32}. The functions of Stm1254 and Stm1263 are both unknown. Stm1254 is predicted to be an outer membrane lipoprotein, and Stm1263 to be a periplasmic protein and neither are referred to in the literature. Transcription of all four of these genes was significantly upregulated after overexpression of σ^E (chapter 5). This chapter looks at the effects of deleting these genes individually, and also at combining *stm1250* and *stm1251* deletions. Through

collaboration with the Kormanec group we also discuss regulation of these genes, and suggest future work on this interesting genomic locus.

9.2 Genomic Locus of this Putatively RpoE Regulated Islet



After blasting all of the genes in the region against the non redundant database using the fragment viewer in colibase (Chaudhuri, Khan, and Pallen, 2004), only two of the genes (*stm1253* (inner membrane protein) and *stm1263* (periplasmic protein)) had homologues in *E. coli* K12. *stm1255* through to *stm1259* were all ~80% similar at the DNA level to *Yersinia* species. ABC transporters, *stm1250* and *stm1251* are as described in the introduction specific to *Salmonella*, with one of the other putatively σ^E regulated genes, *stm1254* (outer membrane lipoprotein) only having distant relatives (~35% identify at the protein level) in *Helicobacter hepaticus* and *Leptospira interrogans.* Also of particular interest are *stm1247* and *stm1262*, both of which are tRNA molecules and may indicate that they have been acquired by horizontal transfer.

9.3 Regulation of *stm1250* and *stm1251*

For three of the four genes discussed in this chapter we have identified putative σ^E regulated promoters by our promoter consensus search, as yet we have not identified the equivalent for *stm1263*. Using S1 mapping the Kormanec group identified two promoters regulating *stm1251* expression, P1 and P2. P1 is located 400bp upstream of the start codon, and is σ^E dependent, as it is highly induced during the established *rpoE* inducing conditions of stationary phase growth and cold shock (Miticka *et al.*, 2003), and absent in a *S.* Typhimurium *rpoE* mutant. P2, which is only 60bps upstream of the start codon, is present in both the WT and *rpoE* mutant strain and is highly induced under heat shock conditions. The sequence of this promoter maps identically to that proposed by Tomayasu et al, (Tomoyasu *et al.*, 2003) who predicted this to be a σ^H dependent promoter. After comparing this sequence to a σ^H dependent promoter consensus (Ramirez-Santos *et al.*, 2001), and in the light of strong induction under heat shock, we would agree that the P2 promoter is probably regulated by σ^H. We therefore propose that *stm1251* is the first gene to be regulated by both σ^E and σ^H dependent promoters. S1 mapping of *stm1250* only identified a single promoter, which is absent in the *rpoE* mutant. Both σ^E dependent promoters contain a 16bp spacing region between the -35 and -10 which is characteristic of σ^E dependent promoters (Miticka *et al.*, 2003;Miticka *et al.*, 2004). We have also constructed a *lacZ* fusion to the 500bp upstream region of *stm1251*, p*stm1251P*. The data from this fusion confirms that seen in S1 mapping (figure 47) in that during exponential phase growth (3 hours) activity of this fusion is the same in both wild type and *rpoE* mutant *S.* Typhimurium backgrounds. However, during stationary phase (8 hours) an increase in activity of this fusion is seen in the wild type strain background that is absent in the *rpoE* mutant. As Stm1251 is a

putative chaperone, we were intrigued to see if lack of this protein resulted in a σ^E activating environment, as occurs in the absence of Skp or SurA. However, activity of the autoregulated *rpoEP3* promoter in the Δ*stm1251* and the Δ*stm1250* backgrounds is similar to that in the wild type strain background.

9.4 Construction and Confirmation of Single Mutants

Each single mutant discussed in this chapter was constructed by lambda red mutagenesis, resulting in deletion of the CDS of the relevant gene and insertion of a kanamcyin cassette from pKD4. All oligonucleotides both for mutant construction and also for mutant confirmation of kanamcyin resistant colonies are tabled in the materials and methods chapter. Insertion of the cassettes was confirmed by comparing the size of PCR amplicons from wild type and putative mutant strains (figure 48).

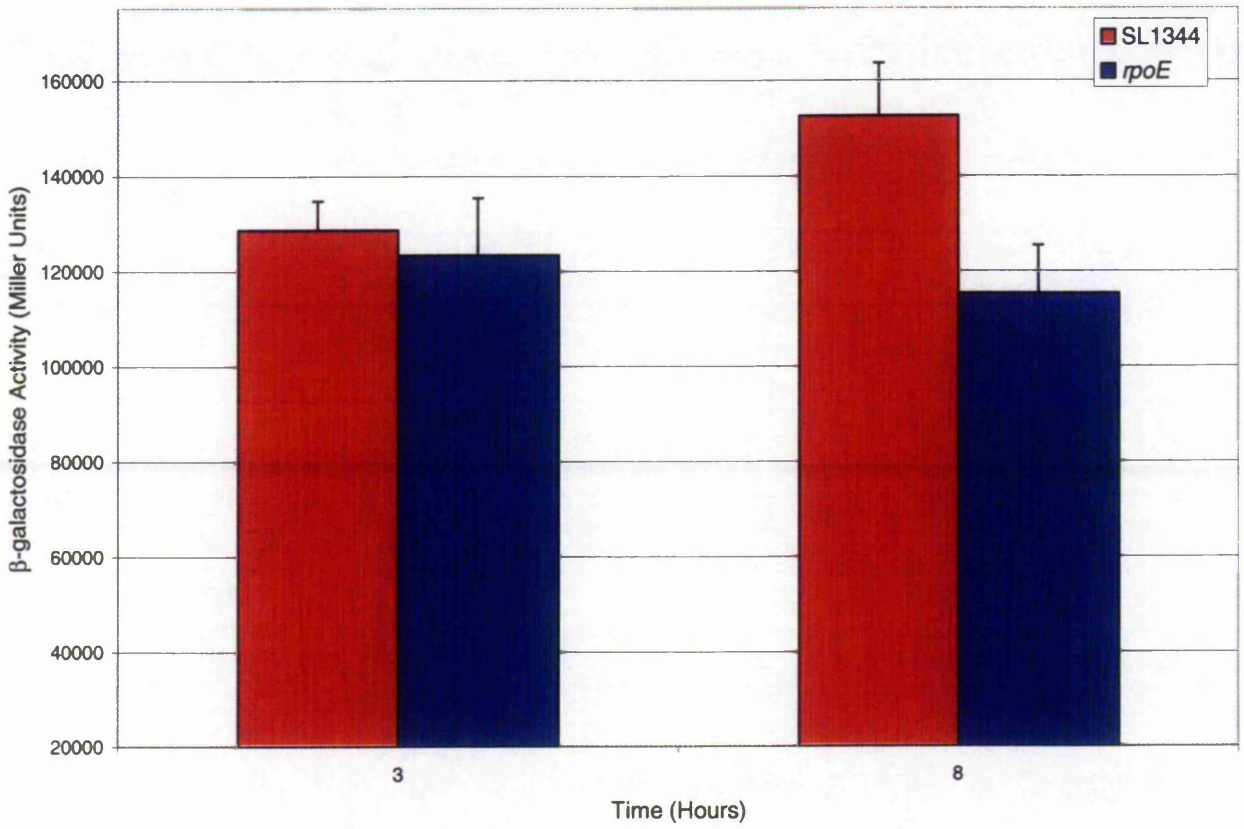


Figure 47. Activity of *pstm1251P* in wild type and Δ*rpoE S.* Typhimurium.
Cultures were grown overnight and then diluted 1/100 into fresh LB medium. β-galactosidase assays were performed at the time points shown, and the activity determined using the arbitrary but well recognized Miller units(Miller, 1972). Each point represents the mean of seven assays, and error bars indicate the standard deviation from the mean.

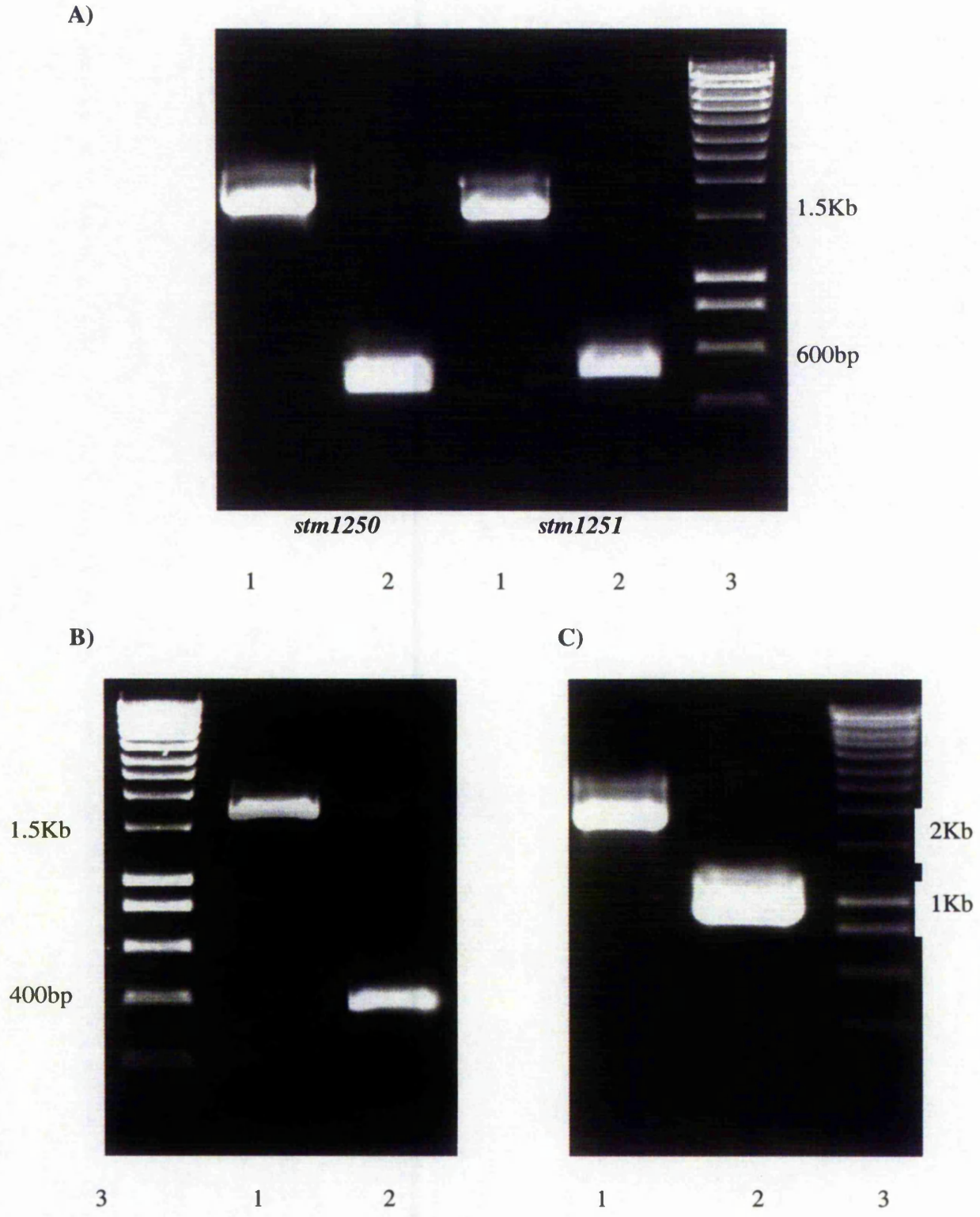


Figure 48. PCR confirmation of mutagenesis.
Agarose gel pictures shown here are confirmation of **A)** Δ*stm1250* and Δ*stm1251*, **B)** Δ*stm1254* and **C)** Δ*stm1263* deletion mutants. Each 1% TBE gel contains a band corresponding to mutant (1) and wild type (2) from an SL1344 background, and a DNA ladder (3).

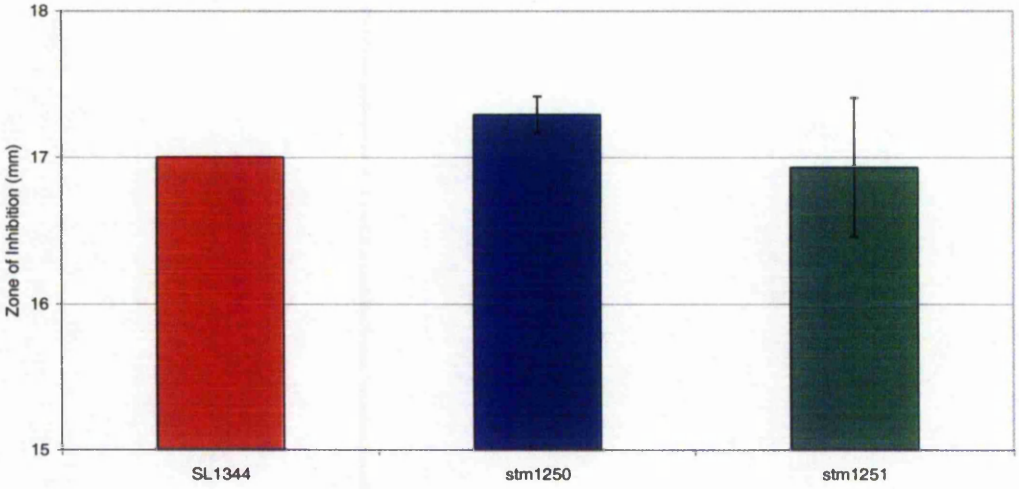
9.5 Mutations in *stm1250* and *stm1251* have No Affect on Growth in L-broth Cultures.

Growth curves of wild type *S.* Typhimurium SL1344, Δ*stm1250*, Δ*stm1251* and a Δ*stm1250* Δ*stm1251* double mutant were compared at 37°C, 42°C and 48°C as well as in the presence of 3% ethanol. Under these conditions, no significant difference between these strains could be observed (GR, data not shown).

9.6 Sensitivity to Polymixin B and Hydrogen Peroxide

Polymixin B is an antimicrobial peptide which binds to the lipid A core of lipopolysaccharide (LPS). After this binding the inner and outer membranes are permeabilised, which results in cell death. We have previously shown that strain GVB311, an *S.* Typhimurium *rpoE* mutant, is significantly more sensitive to killing by polymixin B (Humphreys *et al.*, 1999). Figure 49 shows the comparative sensitivity of Δ*stm1250*, Δ*stm1251* to 100U polymixin B in a disk diffusion assay. There is no significant difference ($p>0.05$, ANOVA) between the sensitivities of these mutant strains and the wild type. We, and others, have demonstrated the importance of σ^E regulated genes in defence against oxidative stress (Humphreys *et al.*, 1999;Testerman *et al.*, 2002). Figure 49 depicts a similar assay as above, using the same strains, but this time the noxious agent tested is 3%H_2O_2. Δ*stm1251* ($p<0.05$) and Δ*stm1250* ($P<0.001$) are both significantly more sensitive to oxidative stress than the wild type strain.

A) Polymixin B



B) Hydrogen Peroxide

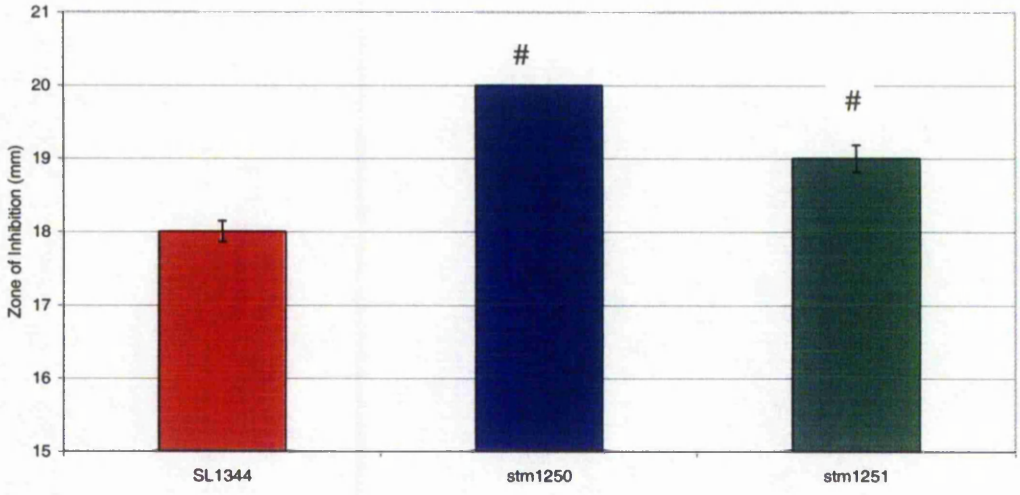


Figure 49. Effect of *Δstm1250* and *Δstm1251* mutations on the sensitivity of *S.* Typhimurium to A) 100U polymixin B and B) 3% hydrogen peroxide.
Each bar represents the mean of 16 disc diffusion replicates and the error bar the standard deviation from the mean. # equals significantly different from SL1344 ($P<0.05$, one way ANOVA).

9.7 Intramacrophage Survival of Δ*stm1250*::kan and Δ*stm1251*::kan

Using the murine macrophage cell line, RAW 264.7, we compared the invasive ability of wild type *S.* Typhimurium to Δ*stm1251* and Δ*stm1250*. Using an MOI ~1, we found that at both 3 and 24 hours post infection there was no significant difference in the recovery of intracellular bacteria between the wild type strain, and the Δ*stm1250* and Δ*stm1251* single mutant strains (figure 50).

9.8 Assessment of Δ*stm1250*::kan and Δ*stm1251*::kan Virulence Using a Competition Assay

σ^E is critical for virulence of *S.* Typhimurium and we therefore hypothesise that some regulon members will display a degree of attenuation *in vivo*. We tested whether Δ*stm1250* and Δ*stm1251* fulfilled this hypothesis via competition assays. Approximately $5x10^3$ of the wild type strain and the appropriate mutant strain were inoculated in a 200μl dose via the IP route. The average CFU/organ for spleen and liver, calculated from data obtained from 3 BALB/c mice, is illustrated in figure 51 for WT v *Δstm1250* and figure 52 for WT v *Δstm1251*. A competition index of 1 indicates that the mutant is equally as virulent as the wild type strain. Both Δ*stm1250* and Δ*stm1251* produced indices of ~0.9 and therefore there is no significant difference between the virulence properties of these strains and the wild type strain via the IP route of infection.

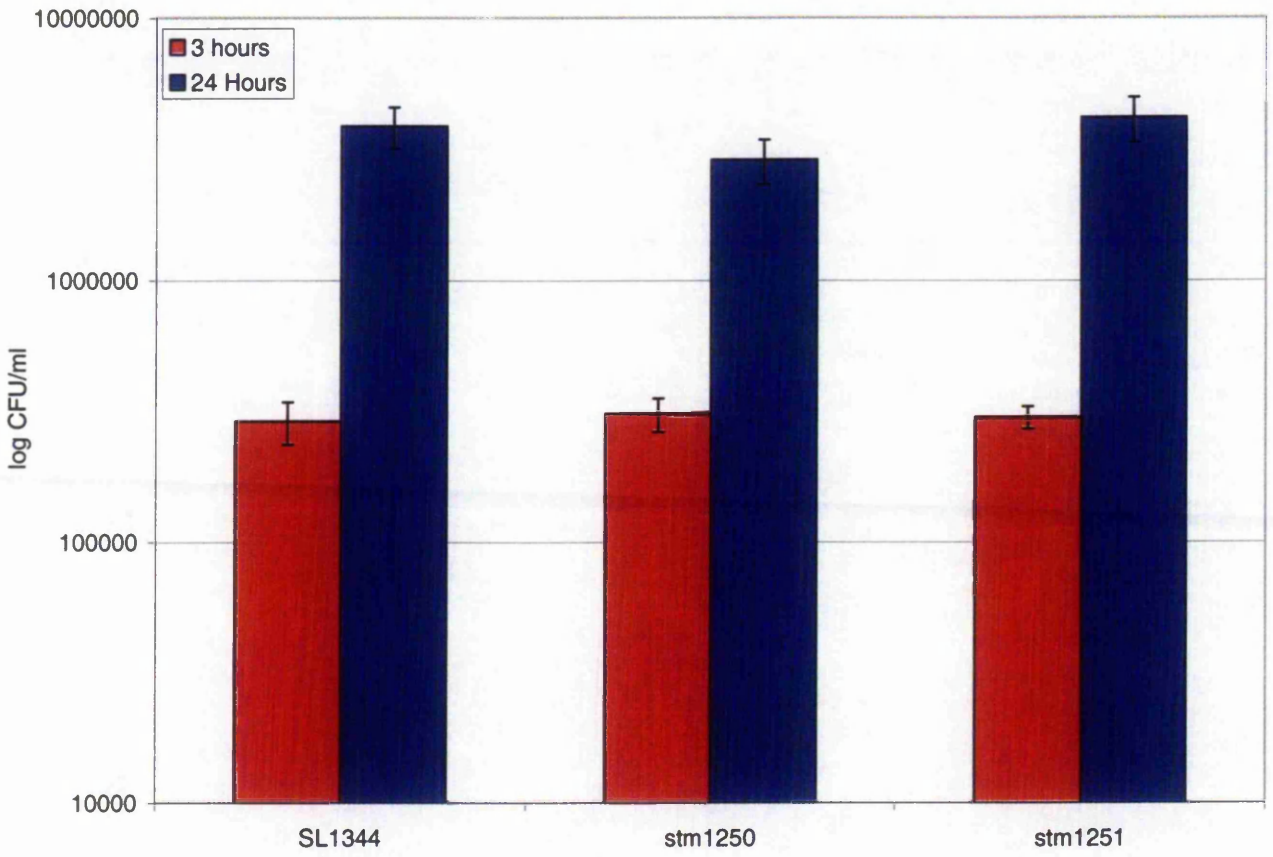


Figure 50. The effect of Δ*stm1250* and Δ*stm1251* mutations on *S.* Typhimurium invasion and survival in macrophages. Bacteria at a multiplicity of infection of ~ 1:1 were incubated with the murine macrophage cell line RAW 264.7. The assay was performed as described in the text. The graph shows the number of viable bacteria (corrected for dose) inside the macrophage at 3 h and 24 h after infection. Each bar represents the mean from triplicate experiments and the error bar indicates the SD.

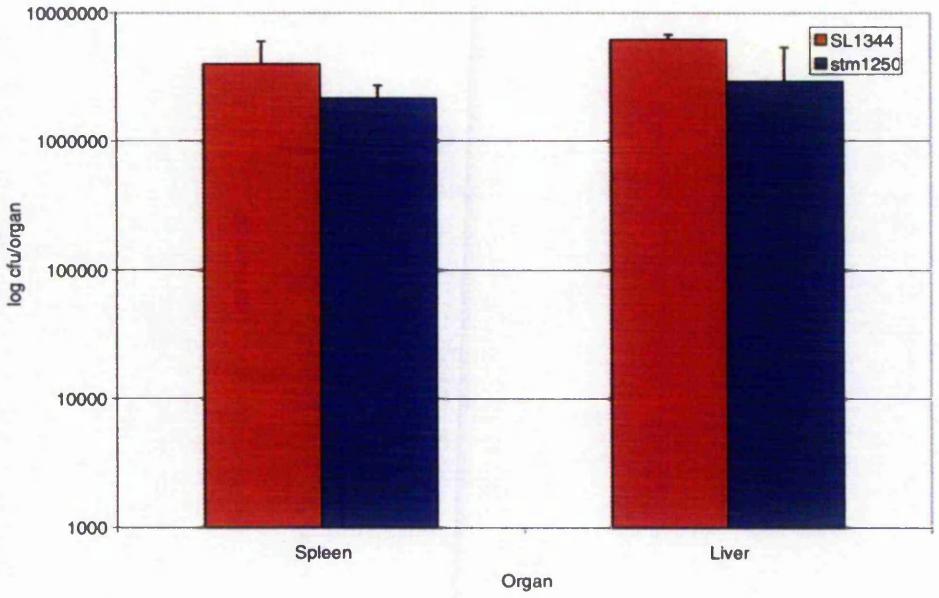


Figure 51. An IP competition assay of wild type *S.* Typhimurium SL1344 v *Δstm1250*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.9, and therefore *Δstm1250* is not significantly different to the wild type (p>0.05, student t test).

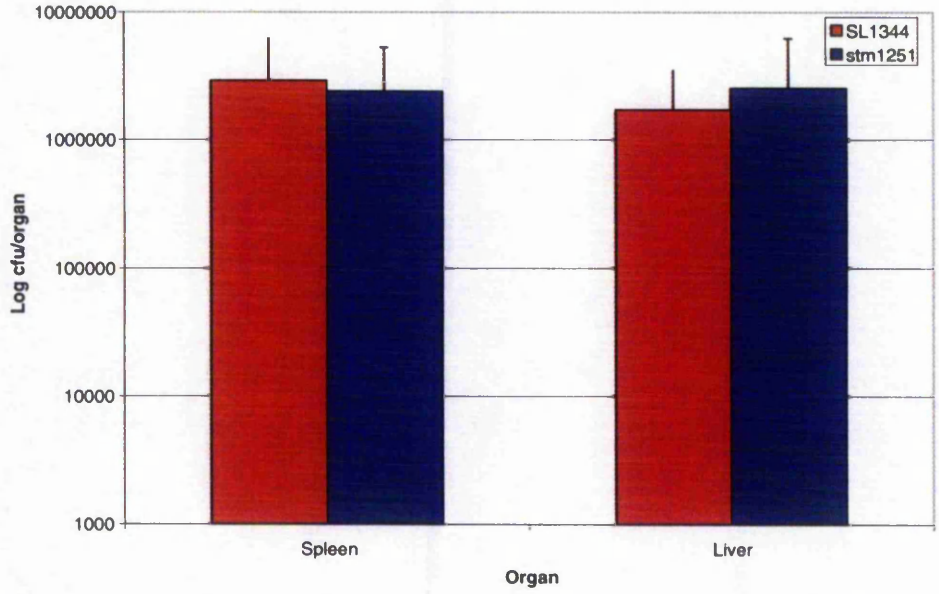


Figure 52. An IP competition assay of wild type *S.* Typhimurium SL1344 v *Δstm1251*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.9, and therefore *Δstm1251* is not significantly different to the wild type (p>0.05, student t test).

9.9 Construction and Confirmation of a Δ*stm1250* Δ*stm1251*::kan Double Mutant

Unlike the other double mutants created during this study we were unable to create a *Δstm1250 Δstm1251::kan* double mutant via P22 transduction. The kanamcyin cassette was removed from *Δstm1250::kan*, using plasmid pCP20, to leave an unmarked deletion, which was then infected with a P22 lysate propogated on the *Δstm1251::kan* strain. The resulting strain after transduction should have been the desired double mutant, but only a single Δstm1251::kan mutant was obtained. This was due to a wild type copy of *stm1250* also being introduced onto the chromosome from the phage, due to the close proximity and relative small size of these genes. As an alternative to P22 transduction we electroporated the lambda red helper plasmid, pKD46, into the unmarked *Δstm1250* strain, and then used red mutagenesis to delete the coding sequence of *stm1251* in this strain, resulting in the desired *Δstm1250 Δstm1251::kan* double mutant (figure 53).

9.10 Effects of the *S.* Typhimurium Δ*stm1250* Δ*stm1251*::kan Double Mutant on Oral Infection of Mice

Although the *stm1250* and *stm1251* mutations had no affect on the ability of *S.* Typhimurium to cause systemic infection of BALB/c mice, we were keen to see if there was any degree of attenuation after oral administration. Although there was a difference in dose (~$5x10^5$ WT v ~$8x10^6$ for mutants) the CFU/organ in both the liver and spleen for both of the single mutants were significantly lower than that of the WT parent, SL1344 ($P<0.05$, unpaired t test with Welch Correction) (figure 54). Preliminary data from the *Δstm1250 Δstm1251::kan* double mutant is that, at least *in vivo* after oral (figure 54) and systemic (GR, data not shown) administration it behaves in most

respects like a *Δstm1250* single mutant, although there was a slight, but not significant (P>0.05, unpaired t test with Welch Correction) difference between the double mutant and the single Δ*stm1250* mutant in the spleen and Peyer's patches.

9.11 Characterisation of Δ*stm1254::*kan and Δ*stm1263::*kan *S.* Typhimurium Mutants.

Neither of these mutants display any differential phenotype compared to the wild type parent strain, SL1344 when tested for sensitivity to polymixin B, hydrogen peroxide and growth at high temperature (GR, data not shown). Figures 55 and 56 depict the average CFU/organ for spleen and liver after competition assay against the wild type strain via the IP route, for Δ*stm1254* and Δ*stm1263* respectively. There is no significant difference (p>0.05, student t test) between the CFU/organ isolated for the WT strain and either of the mutants.

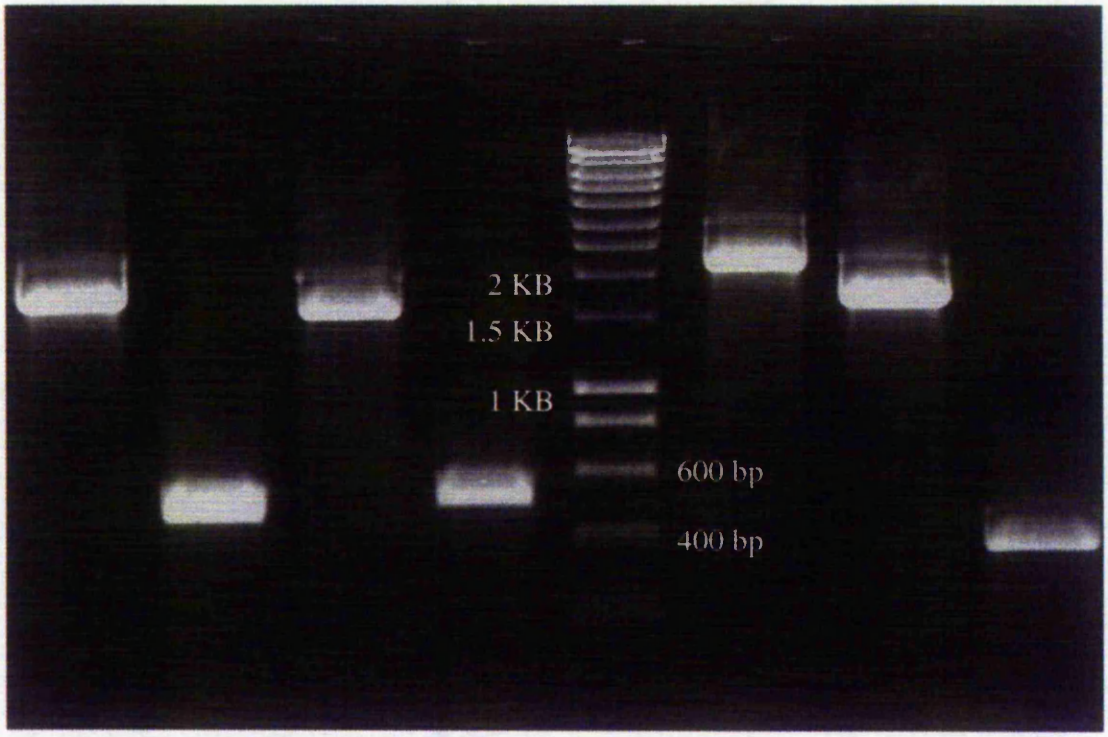


Figure 53. PCR confirmation of mutagenesis.
This 1% TBE gel contains the corresponding bands from WT and mutant for the single mutants of *stm1250* and *stm1251* (1-4), as well as for the *stm1250 stm1251*::kan double mutant (7) with respect to the single *stm1251*::kan (6) and unmarked *stm1250* (8) mutants. The oligonucleotides used for this analysis are also listed.

Lane 1 = Δ*stm1250*::kan
Lane 2 = WT
(Using oligonucleotides stm1250EXTF and stm1250EXTR)
Lane 3 = Δ*stm1251*::kan
Lane 4 = WT
(Using oligonucleotides stm1251EXTF and stm1251EXTR)
Lane 5 = Hyperladder I (Bioline)
Lane 6 = Δ*stm1251*::kan
Lane 7 = Δ*stm1250* Δ*stm1251*::kan
Lane 8 = Δ*stm1250*
(Using oligonucleotides stm1250EXTF and stm1251EXTR).

All oligonucleotides used are listed in the materials and methods.

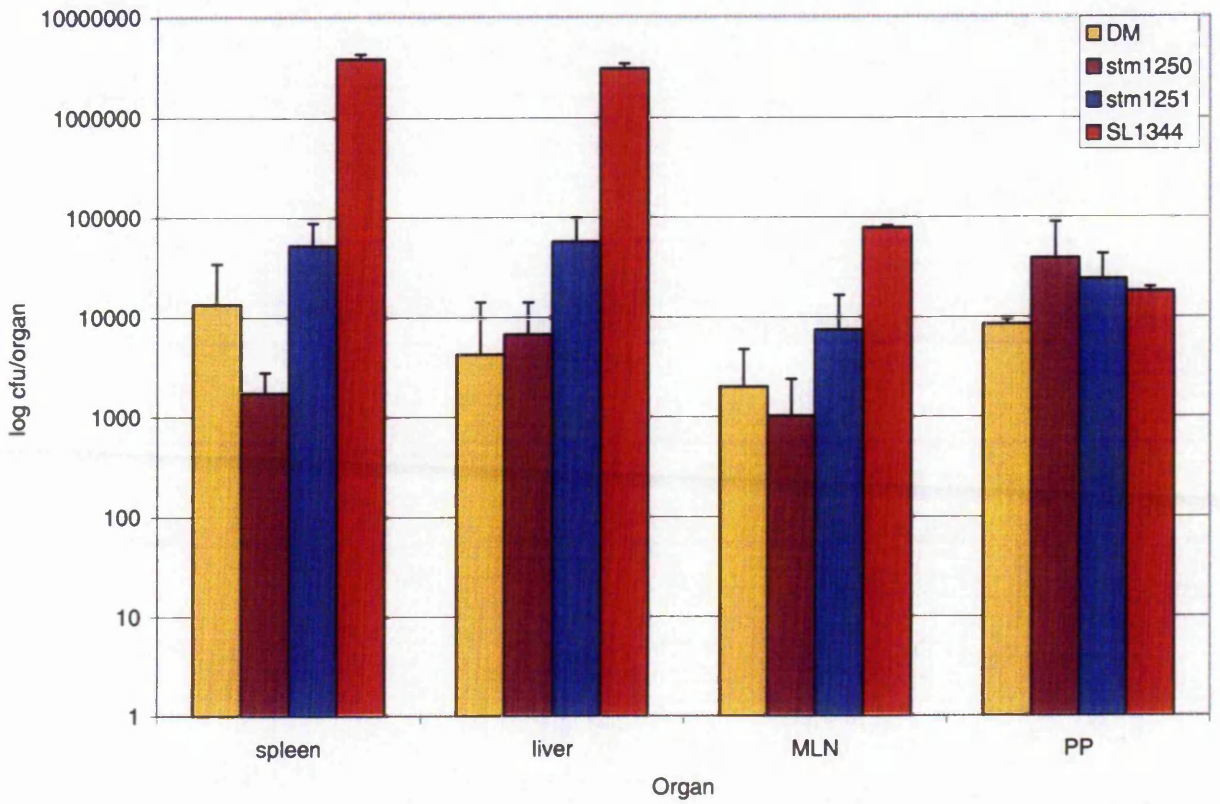


Figure 54. Effect of *Δstm1250::kan* , *Δstm1251::kan and Δstm1250Δstm1251::kan* (DM) mutations on the ability of *S.* Typhimurium to infect mice via the oral route.

Mice were inoculated orally with ~$5x10^5$ CFU of the WT or ~$8x10^6$ of the mutant strain. The number of bacteria present in different organs were determined 5d later. The bar represents the mean of 5 mice and the error bar indicates the SD. PP, Peyer's patches: MLN, mesenteric lymph node.

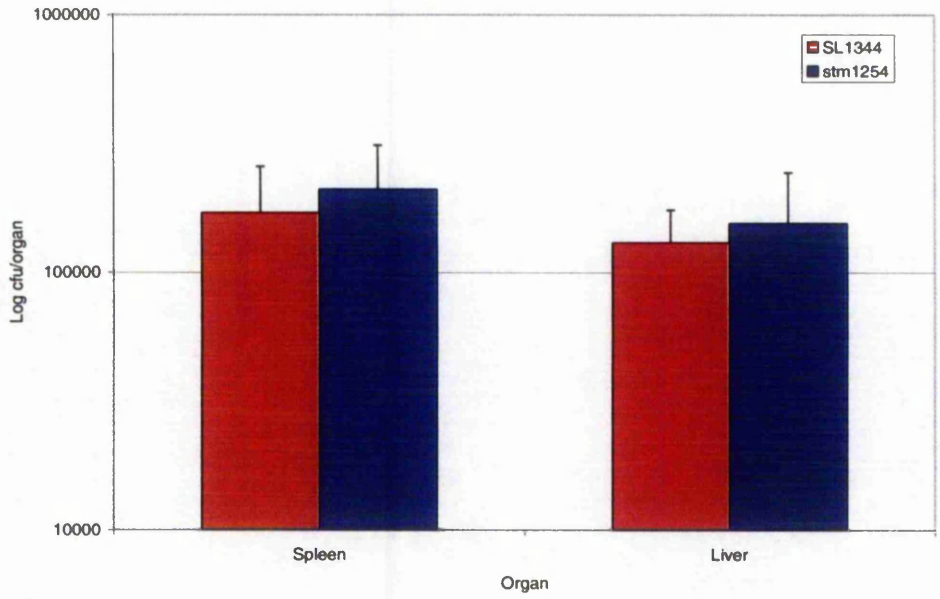


Figure 55. An IP competition assay of wild type *S.* Typhimurium SL1344 v *Δstm1254*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of ~1, and therefore *Δstm1254* is not significantly different to the wild type strain ($p>0.05$, student test).

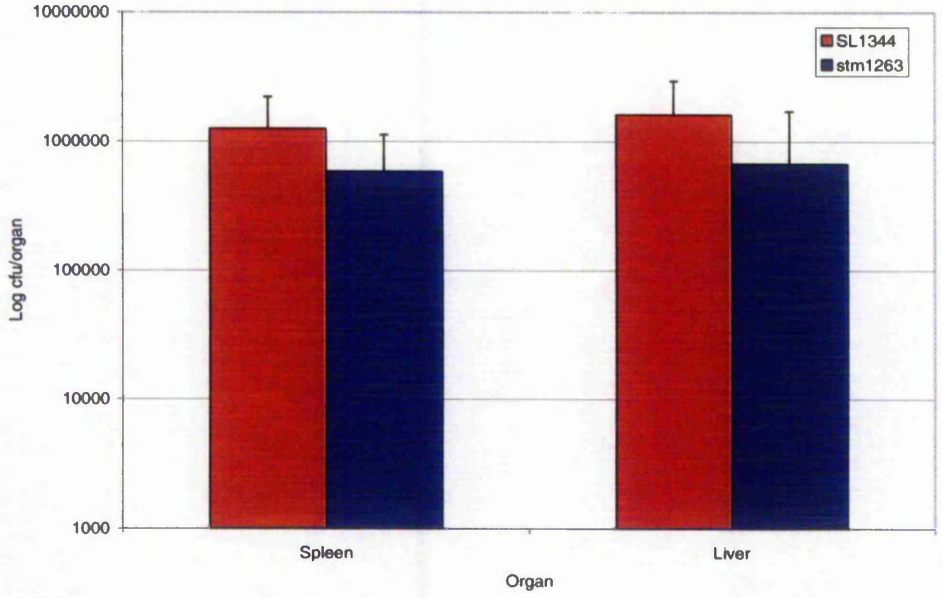


Figure 56. An IP competition assay of wild type *S.* Typhimurium SL1344 v *Δstm1263*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.42, and therefore *Δstm1263* is not significantly different to the wild type strain ($p>0.05$, student t test).

9.12 Discussion

Expression of *stm1251* (*agsA*) was hypothesised to be regulated by the alternative sigma factor, σ^H (Tomoyasu *et al.*, 2003). In this study we have identified *stm1251* as also being σ^E regulated. To our knowledge this is the first gene to be regulated by both these alternative sigma factors. In this study we have also shown the gene upstream of *stm1251*, *stm1250*, as being σ^E regulated. *stm1250* and *stm1251* are the first, thus far confirmed *Salmonella* specific σ^E regulated genes.

Microarray analyses of other groups have implicated roles for *stm1250* and *stm1251* within a number of different environments. Transcription of both genes has been shown to be upregulated by oxidative stress and in stationary phase (Porwollik *et al.*, 2003), *stm1251* is upregulated by the macrophage intracellular environment (Eriksson *et al.*, 2003), and *stm1250* is upregulated by the addition of polymixin B to the growth media (Bader *et al.*, 2003), all of which are known inducing conditions of the σ^E regulon. We thought that co-regulation of *stm1251* by both σ^E and σ^H may be indicative of an essential role for this gene product under certain conditions. In the majority of the assays and conditions tested in this study, individual deletions of either *stm1250* or *stm1251* have only minor affects on *S.* Typhimurium. There are two exceptions to this, firstly in agreement with (Porwollik *et al.*, 2003) both Δ*stm1250* and Δ*stm1251* are significantly more sensitive to oxidative stress than the WT strain. Secondly, both the Δ*stm1250* and Δ*stm1251* strains are significantly impaired in their ability to survive in a murine host after oral infection, although the greatest difference was seen between Δ*stm1250* and the WT strain. We know that this is not through its inability to survive and replicate within the spleen and liver from the results from the IP experiments. It

appears that the mutants can invade and colonise the Peyer's patches normally but they are either defective in translocation from here to the MLN or in surviving within the MLN environment, both of which require further investigation. The double deletion mutant behaves similarly to a Δ*stm1250* single mutant implicating the greater role for *stm1250* when compared with *stm1251*.

Activity of the autoregulated *rpoEP3* promoter in both the *stm1250* and *stm1251* deletion strains is comparable to that seen in wild type *S.* Typhimurium, indicating that loss of function of their products does not result in an σ^E inducing environment. We considered that this may be due to functional overlap between these two genes; however there is no increase in activity of the *rpoEP3* fusion in the Δ*stm1250*Δ*stm1251* double mutant. This is in agreement with these proteins having a cytoplasmic location and thus their absence not resulting in envelope stress.

We were intrigued by the number of putatively σ^E regulated genes within such a small region of the *S.* Typhimurium chromosome, and constructed deletion mutants of *stm1254* and *stm1263* also. Neither of these genes demonstrated a significant phenotype under the conditions tested. The next step in the investigation of this genetic locus is to try and delete the entire region from *stm1250* through to *stm1263* and to assess the phenotypic difference this makes both *in vitro* and *in vivo*. A precise dissection of the role that the *Salmonella* specific genes, *stm1250* and *stm1251* play in the oral route of infection should also be performed, as well as analysis of these mutants in chicken and calve infection models. At this point the question still remains as to what part in the stressful *Salmonella* life cycle these genes play and why they are both σ^E and in the case of *stm1251*, σ^H regulated.

Chapter 10 - A Compendium of the Remaining Mutants Constructed

10.1 Introduction

The data presented in this final results chapter is a compendium of the data we have obtained thus far for the remaining 14 mutants constructed. They have been grouped thus because at this stage they show no or limited phenotype either *in vitro* or *in vivo* under the conditions tested. The majority of the genes discussed in this chapter are of unknown or putative function and there is limited or no reference to them in the literature. Some of the genes however have been discussed briefly and they will be introduced individually. Also where significant sequence or protein domains have been discovered through bioinformatical analysis, this is also discussed. All reference to PFAM domains is taken from Bateman *et al.*, 2004.

yccV in *E. coli* has been identified as a hemimethylated DNA binding protein that negatively controls *dnaA* expression (d'Alencon *et al.*, 2003), the regulatory region of *yccV* contains two DnaA boxes, 3 dam (DNA adenine methylation) sites, and five Fis binding sites. Bioinformatical screens can add no extra insight into the function of this protein. In chapter 5 we have shown that *S.* Typhimurium *yccV* is upregulated on the microarrays after overexpression of σ^E, and was also identified by the two plasmid screen, although we were unable to locate a putative σ^E regulated promoter based on our promoter consensus screen. Subsequent to our mutation of this gene the Kormanec group performed S1 mapping and confirmed that transcription from the promoters of *yccV* are unaltered in an *rpoE* mutant. Therefore the σ^E regulation of this gene is indirect and maybe via σ^H.

mlc is another gene like *yccV* in that after subsequent analysis it appears this gene may be indirectly regulated by σ^E through its regulation of σ^H, although S1 mapping is still

to be performed to confirm this. This conclusion has been drawn again from the lack of a putatively σ^E regulated promoter after consensus screening and the fact that in the literature *E. coli mlc* has been assigned a putative σ^H dependent promoter, P2. A dramatic increase in *mlc* expression was observed upon heat shock or in cells over expressing σ^H (Shin *et al.*, 2001). In *S.* Typhimurium the -35 sequence of this promoter is identical but the -10 regions are very different. However, as we have not identified a σ^E regulated promoter for this gene it may well be that *mlc* is indirectly regulated by σ^E through σ^H. The function of Mlc is as a global regulator of carbohydrate metabolism Kim *et al.*, 1999. It forms a member of a group of proteins which include transcriptional repressors, sugar kinases and yet uncharacterised open reading frames known as ROK (repressor, ORF, kinase), which includes other proteins such as the N-acetylglucosamine repressor, NagC, from *E. coli* (Plumbridge, 2001).

The function of *yggN* is unknown. YggN possesses a signal sequence between position 1 and 21 and therefore has been designated as a periplasmic protein, and is also referred to as EcfF (Dartigalongue, Missiakas, and Raina, 2001), where it was identified as an σ^E regulated gene in *E. coli*. We have identified a putative σ^E dependent promoter based on our consensus in *S.* Typhimurium. After comparison of the amino acid sequence of this protein against the non-redundant translated database it is apparent that this protein is not ubiquitous across species, with homologues only identified in species of *Salmonella*, *E. coli*, *Shigella* and *Yesinia*. Another putatively σ^E regulated gene, *yggT*, is located just downstream of *yggN*. Again our promoter consensus search identified a putative σ^E dependent promoter upstream of *yggT*, and this has now been validated by S1 mapping (Kormanec J, pers. comm.). The function of YggT is still unknown but does possess a protein domain repeat which is conserved in hypothetical integral membrane proteins.

The oligopeptide permease system of *S.* Typhimurium and other bacteria is a periplasmic-binding, protein-dependent transport system, which can transport any peptides consisting of between 2 and 5 amino acid residues (Andrews and Short, 1985;Andrews, Blevins, and Short, 1986). This allows the bacteria to utilise small oligopeptides as both carbon and energy sources for growth. The system consists of five proteins, *oppABCD*F. From our array analysis all of the genes in the *opp* system were significantly upregulated although our promoter consensus search identified a promoter upstream of *oppC*. We are awaiting S1 mapping of this region to confirm direct or indirect effects of σ^E. Regulation of the oligopeptide permease system has only been briefly discussed in the literature and not since 1986. However the analysis performed in these studies is consistent with an operon where deletion of *oppA* can have polar effects on *oppB*, *oppC* and *oppD* (Hiles *et al.*, 1987). To look for involvement of the *S.* Typhimurium *opp* genes in virulence we mutated the *oppD* gene to try and minimise polar effects on the other members of the system. *oppD* and *oppF* were both identified in a signature tagged mutagenesis screen of *S.* Typhimurium as potential virulence related genes (Hensel *et al.*, 1995). Future work will involve mutating each member individually and the system as a whole to look for differences in virulence between them and confirmed σ^E regulated *opp* genes. In *Xenorhabdus nematophila*, a pathogen of insects, although *opp* allows the use of peptides as a nutrient source, it is not essential for survival in the host (Orchard and Goodrich-Blair, 2004).

The *bacA* gene (now also called *uppP*), which when overexpressed confers bacitracin resistance (El Ghachi *et al.*, 2004) was originally identified as σ^E regulated in the two plasmid screen of *E. coli* for σ^E regulated genes (Rezuchova *et al.*, 2003). In *S.* Typhimurium we have identified a putative σ^E regulated promoter for this gene which

was also significantly upregulated after overexpression of σ^E determined by microarray analysis. The physiological role of *bacA* has been characterised in *E. coli*, and its function is to catalyse the dephosphorylation of C_{55}-PP, a reaction that generates the essential lipid, undecaprenyl phosphate C_{55}-P, used for the synthesis of various cell wall components (Chalker *et al.*, 2000). In *S. pneumoniae* inactivation of *bacA* leads to a strain displaying hypersensitivity to bacitracin and slightly reduced virulence in a mouse model (Chalker *et al.*, 2000). The *E. coli* equivalent mutant is not however hypersensitive to bacitracin presumably because this bacteria is already more resistant to Gram positive bacteria due to the additional outer membrane.

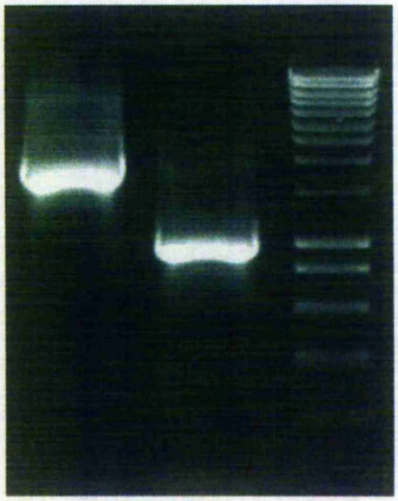
Protease III, encoded for by *ptr*, is a Mg^{2+} dependent endopeptidase located within the periplasmic space, whose known function is to degrade small peptides of molecular mass less than 7 kDa. Mutants lacking protease III activity in *E. coli* have been described but are not apparently phenotypically altered (Finch *et al.*, 1986). As with the majority of genes in this chapter we are the first according to the literature to mutate this gene in *S.* Typhimurium and look for a virulence phenotype.

The remaining 7 genes have no reference in the literature and in the main have putative function based on sequence or protein domain homology. YiaD, an outer membrane lipoprotein belongs to the OmpA family. YgiM is an outer membrane protein containing a SH3b domain, thought to be involved in signalling. YraP is a periplasmic protein containing two BON domains (Yeats and Bateman, 2003;Bateman *et al.*, 2004), which are found in a family of osmotic shock protection proteins such as OsmY. It is also found in some secretins and a group of potential haemolysins. The likely function of the BON domain is attachment of the protein to phospholipid membranes. YabI is a member of the DedA family of proteins which contain multiple transmembrane

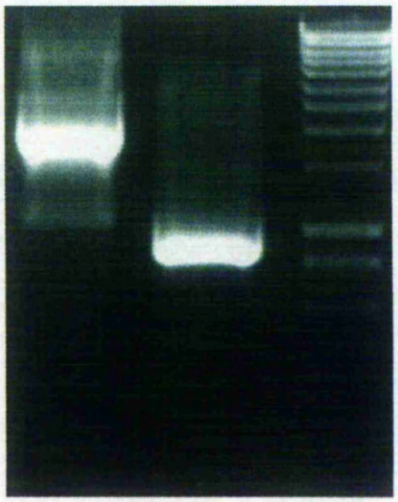
domains, YbbN is a thioredoxin like protein, DedD a putative lipoprotein which is conserved but remains uncharacterised and YrfH is a putative heat shock protein containing a S4 domain, thought to mediate binding to RNA.

All of the genes introduced have been mutated in *S.* Typhimurium 12023 with lambda red mutagenesis and the CDS replaced with a kanamcyin cassette, which afterwards has been transduced into *S.* Typhimurium SL1344 (figure 57). All strain numbers and oligonucleotides used for both mutagenesis and checking of the mutant strains are listed in the materials and methods.

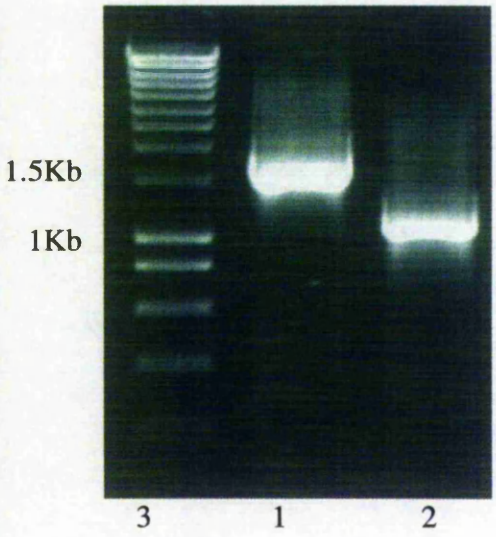
10.2 Confirmation of Mutagenesis by PCR



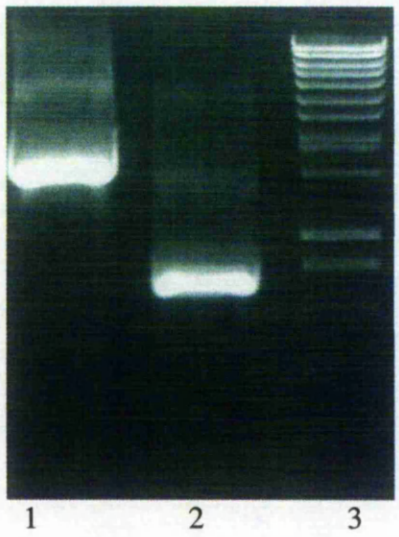
yggN



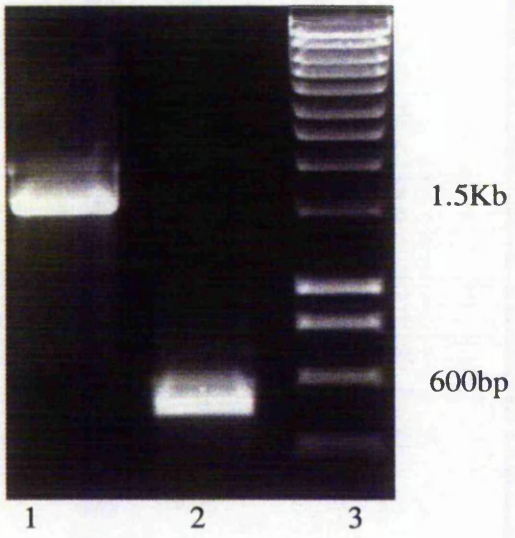
yggT



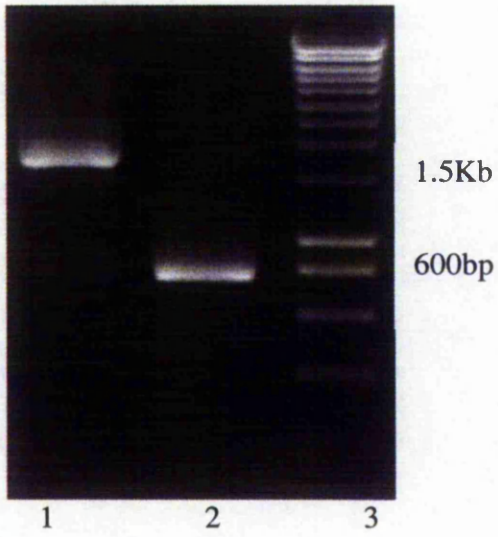
oppD



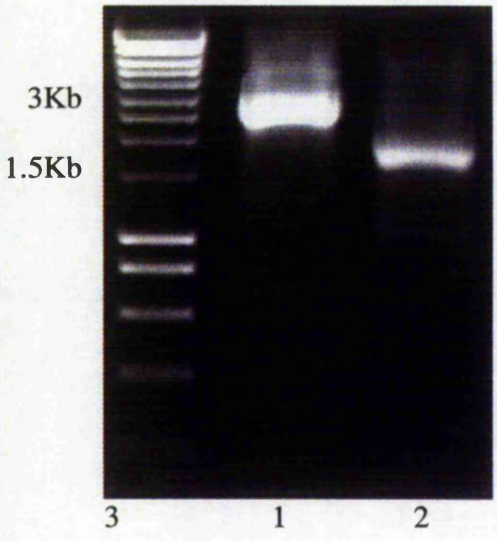
yiaD



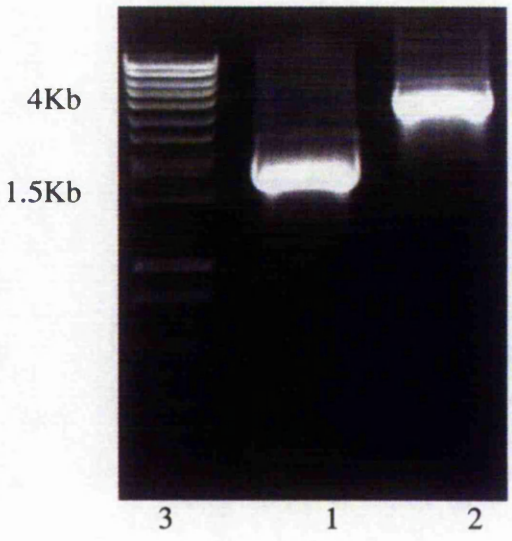
yccV



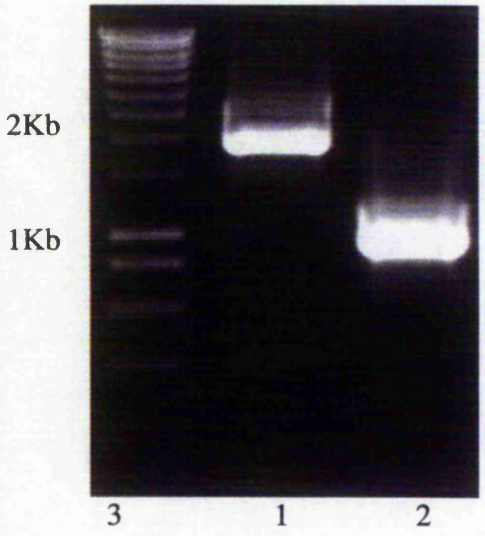
ygiM



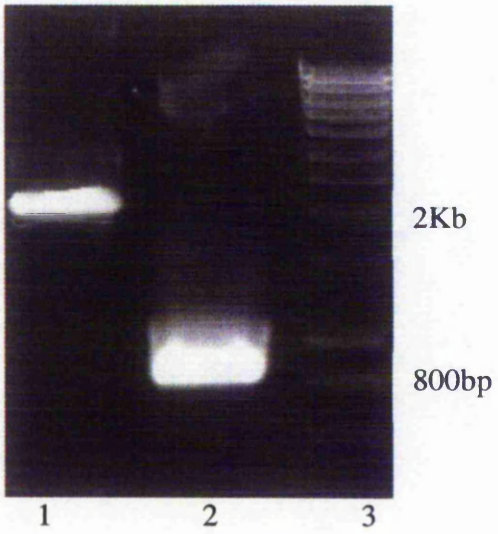
bacA



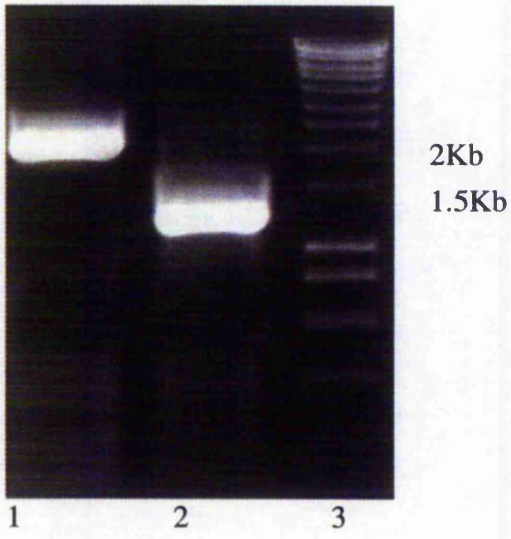
ptr



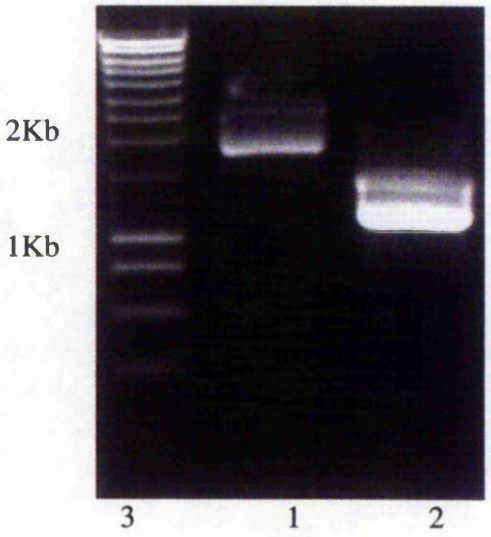
yraP



yrfH



yabI



ybbN

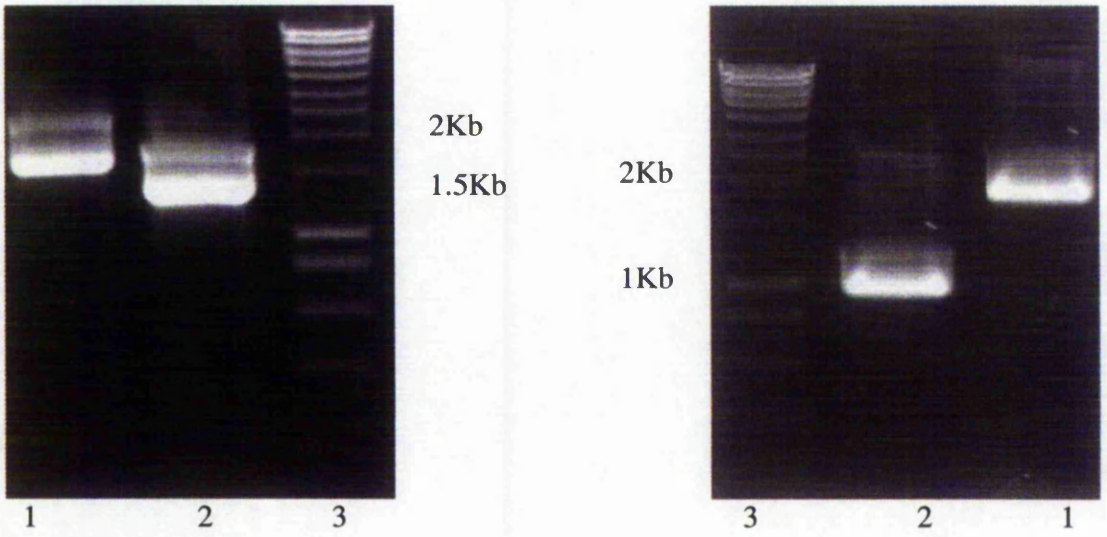


Figure 47. Confirmation of mutagenesis.

After mutagenesis in *S.* Typhimurium 12023 and then again after P22 transduction into SL1344 all mutants were confirmed and reconfirmed by PCR using primers homologous to the regions just out with the 5' and 3' of the CDS mutated. Agarose gel pictures shown here are 1% TBE, each containing a band corresponding to mutant (1) and wild type (2) from an SL1344 background, and a DNA ladder (3).

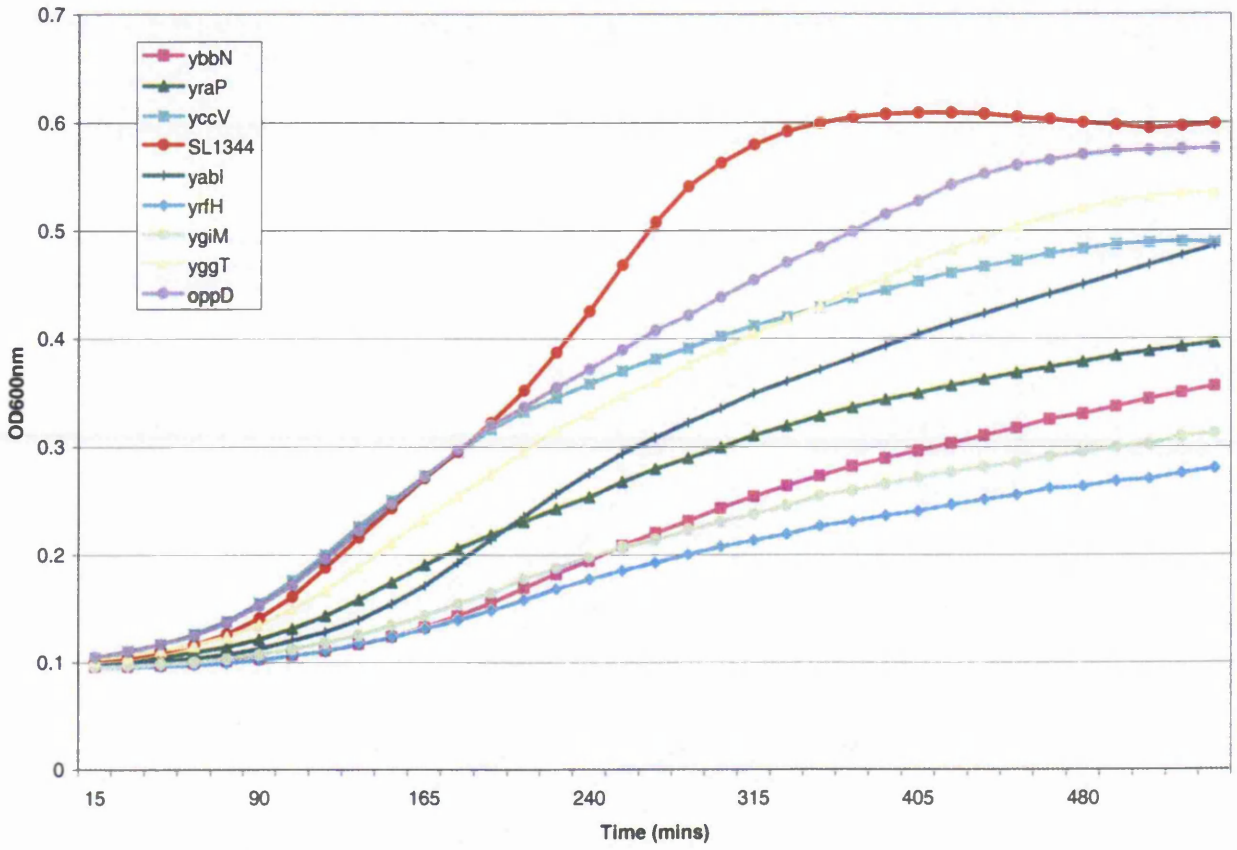


Figure 58. Growth curve of wild type *S.* Typhimurium (SL1344) and eight mutants with a growth defect at 46°C.
Data illustrated here is a mean OD600nm obtained from triplicate curves on the Bioscreen C machine. Standard error mean have been calculated but are too small to be visible on the data points.

10.3 *in vitro* Analysis

All mutants have been assayed for growth in L-Broth at 37°C, 42°C and 46°C, as well as their sensitivities to hydrogen peroxide and polymixin B. None of the mutants show any differential characteristics from the wild type strain under any of these conditions except 46°C where 8 of the mutants demonstrated a reduced growth when compared to the parent wild type strain, SL1344. (Figure 58).

10.4 *in vivo* Analysis Via the IP Route.

All of the mutants discussed in this chapter were tested *in vivo* using competitive index studies. A mixed inoculum of ~$2x10^3$ CFU/200µl dose was administered via the IP route in groups of 3 BALB/c mice. Mice were culled three days post infection and the CFU of each strain in the spleen and liver was determined and the ratio of input versus output of the two strains calculated and reported below as the competition index (CI) (figures 59-70). None of the CFU/organ for any of the mutants, with the exception of *ybbN*, were significantly different (student t test, $P>0.05$) to the wild type strain via this route of infection, in this infection model. *ybbN* however was just significantly different to the wild type strain (student test, $p<0.05$) (figure 69), with an average CI of 0.25.

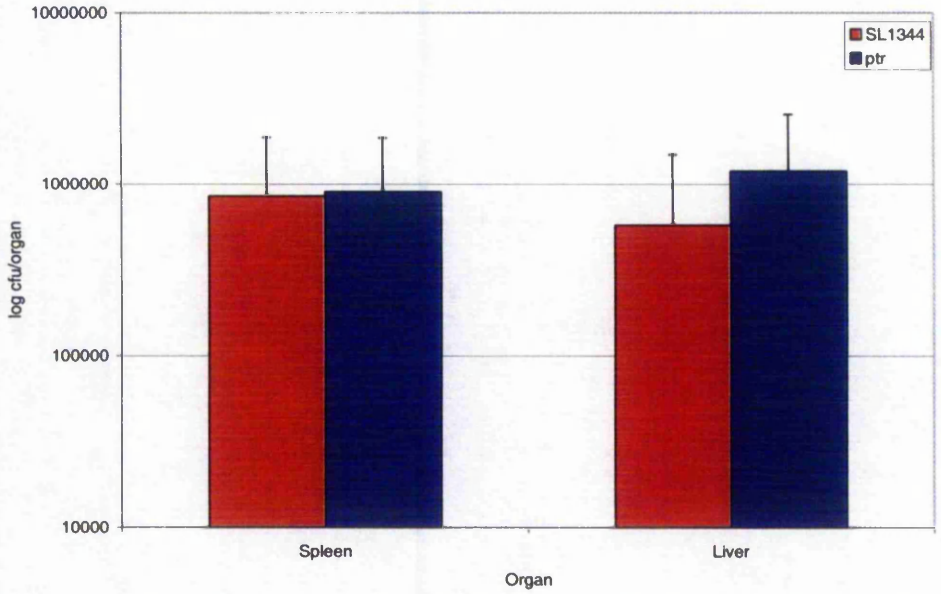


Figure 59. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*ptr*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 1.1, and therefore Δ*ptr* is not significantly different to the wild type strain.

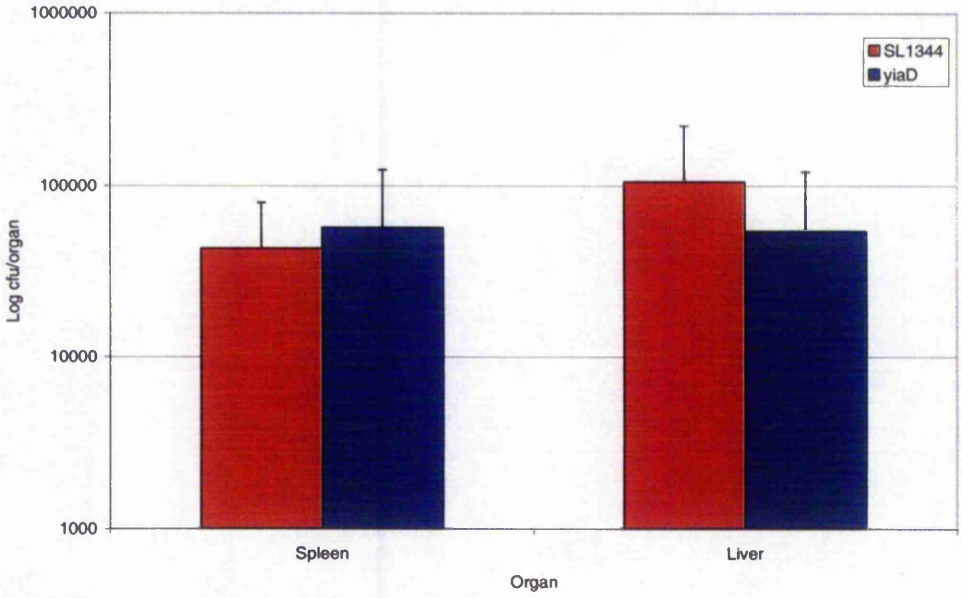


Figure 60. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*yiaD*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.9, and therefore Δ*yiaD* is not significantly different to the wild type strain.

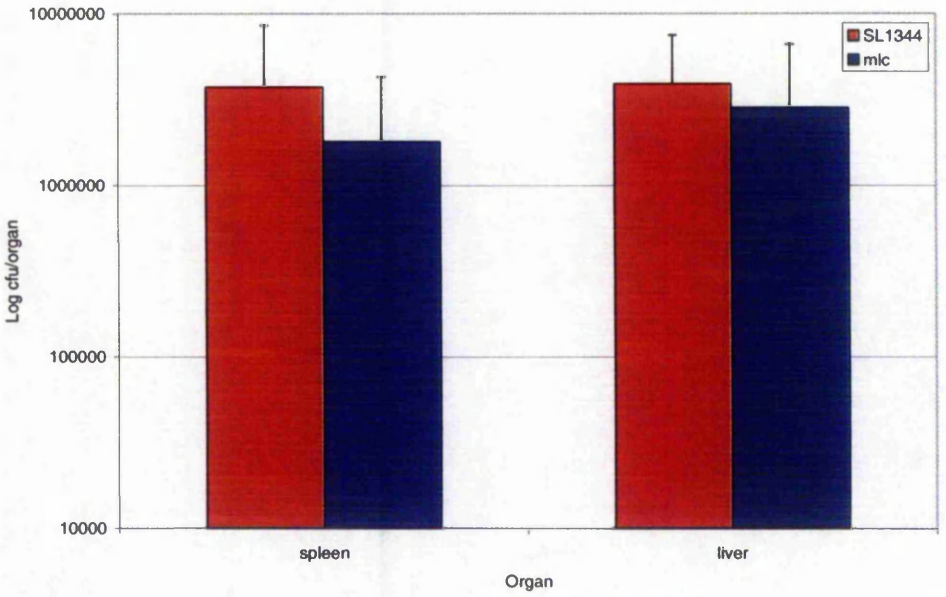


Figure 61. An IP competition assay of wild type *S.* Typhimurium SL1344 v *Δmlc*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.67, and therefore *Δmlc* is not significantly different to the wild type strain.

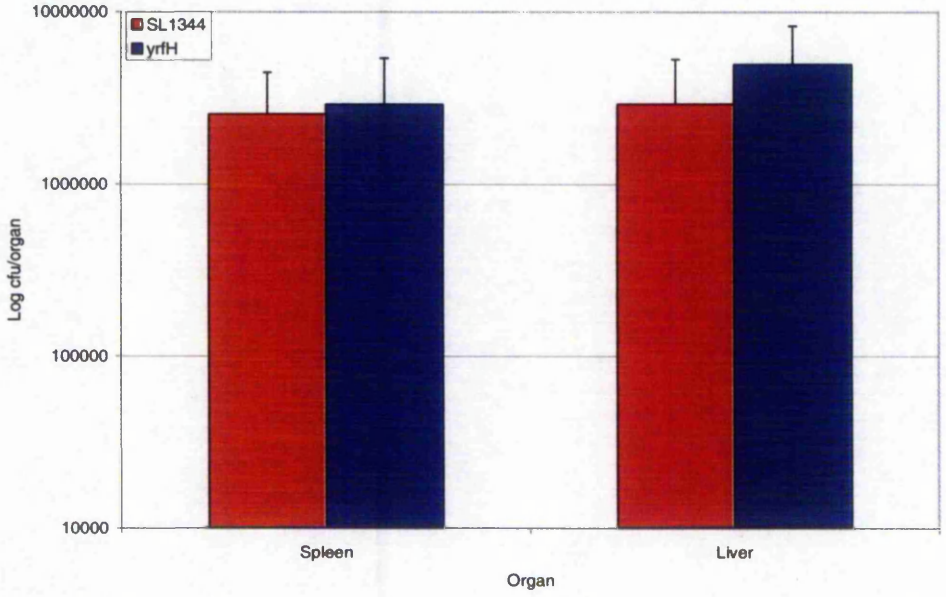


Figure 62. An IP competition assay of wild type *S.* Typhimurium SL1344 v *ΔyrfH*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 1.2, and therefore *ΔyrfH* is not significantly different to the wild type strain.

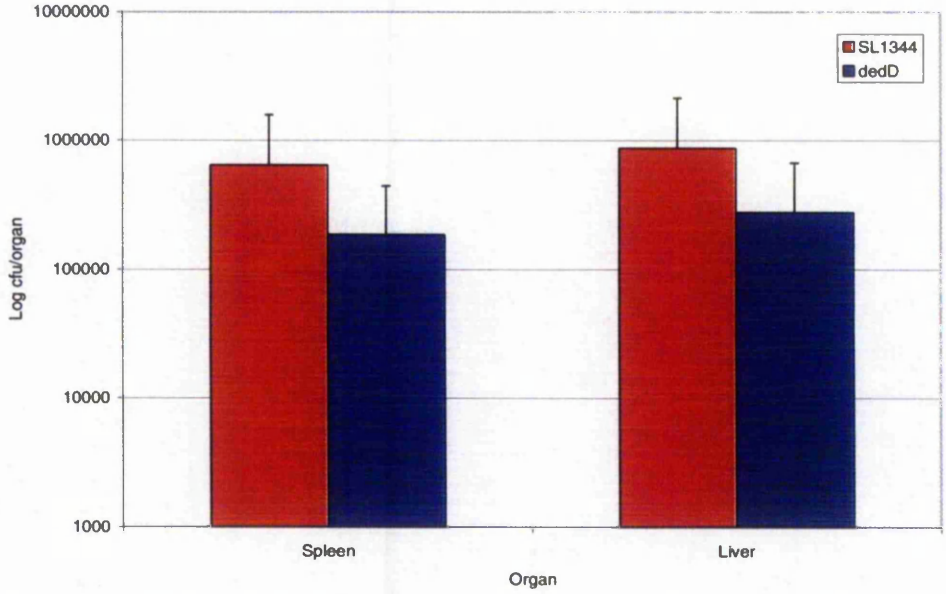


Figure 63. An IP competition assay of wild type *S.* Typhimurium SL1344 v *ΔdedD*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.3, and therefore *ΔdedD* is not quite significantly different to the wild type strain.

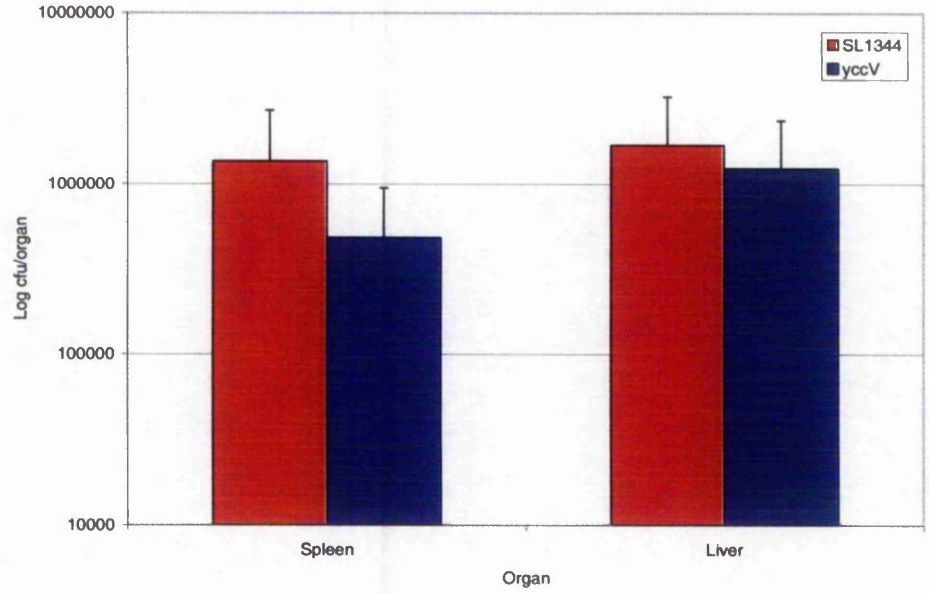


Figure 64. An IP competition assay of wild type *S.* Typhimurium SL1344 v *ΔyccV*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.6, and therefore *ΔyccV* is not significantly different to the wild type strain.

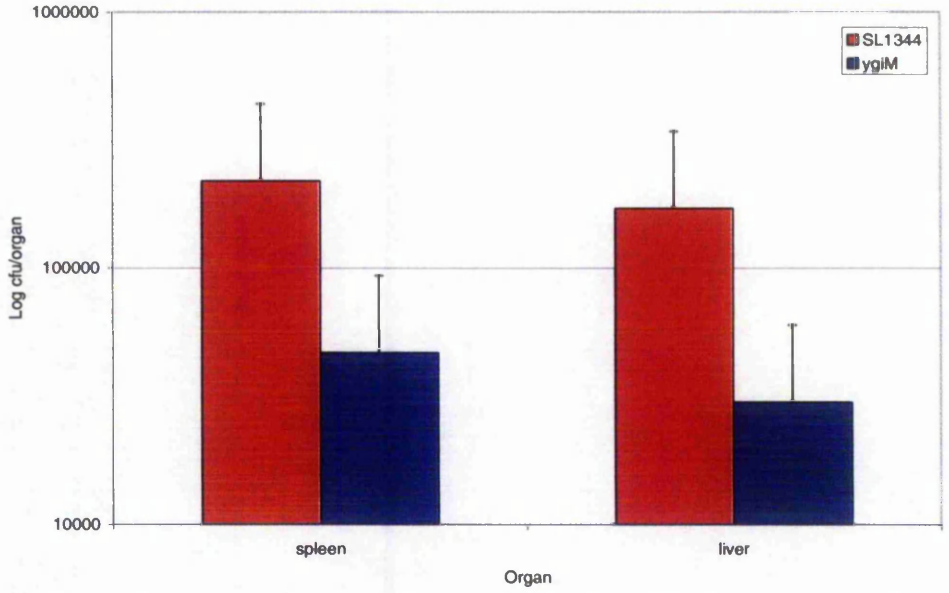


Figure 65. An IP competition assay of wild type *S.* Typhimurium SL1344 v *ΔygiM*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.33, and therefore *ΔygiM* is not significantly different to the wild type strain.

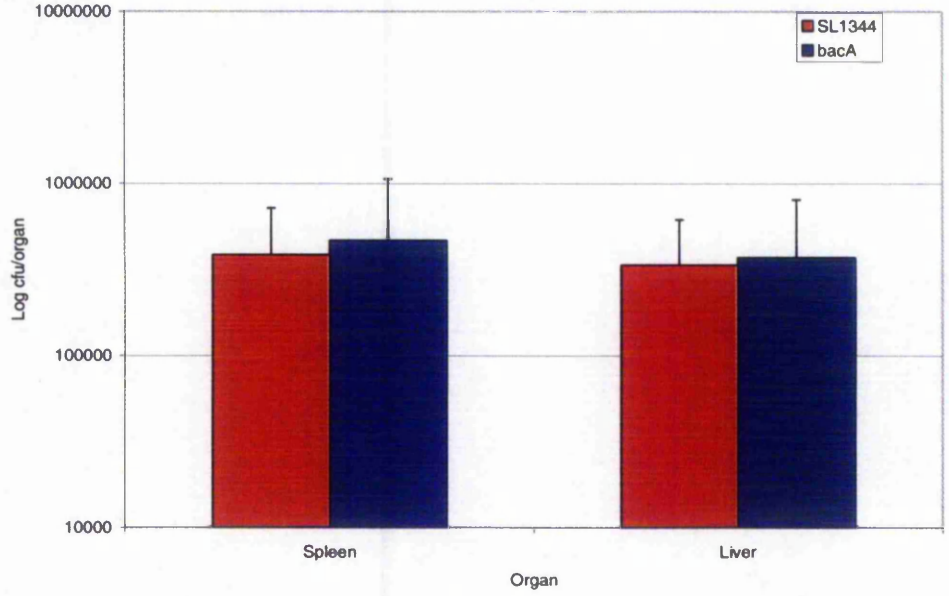


Figure 66. An IP competition assay of wild type *S.* Typhimurium SL1344 v *ΔbacA*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 1.1, and therefore *ΔbacA* is not significantly different to the wild type strain.

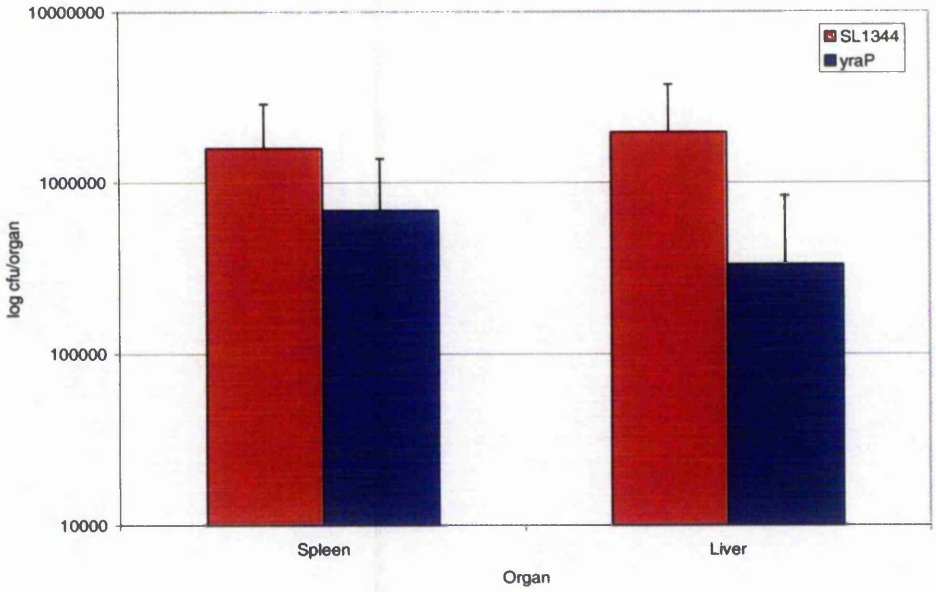


Figure 67. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*yraP*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.39, and therefore Δ*yraP* is not significantly different to the wild type strain.

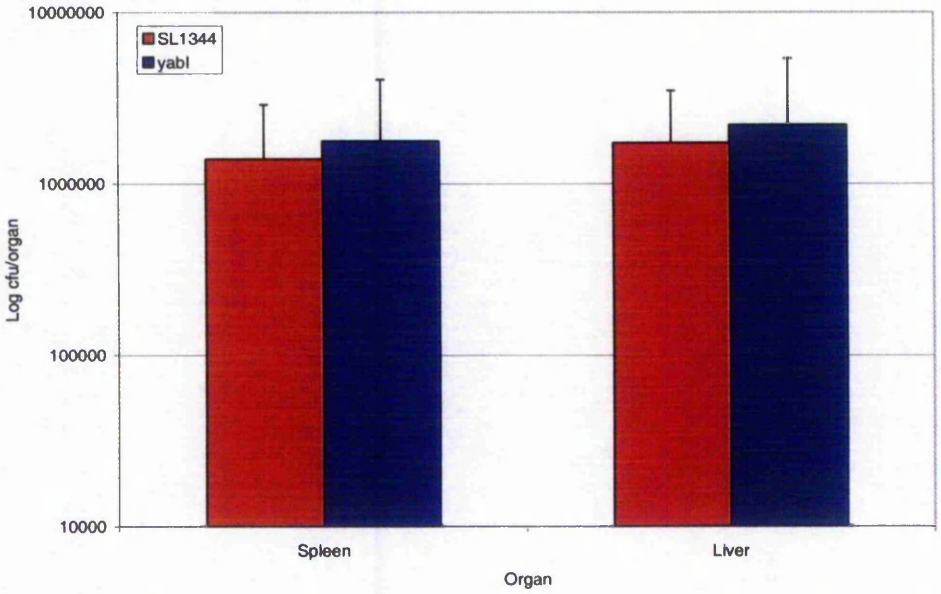


Figure 68. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*yabI*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.89, and therefore Δ*yabI* is not significantly different to the wild type strain.

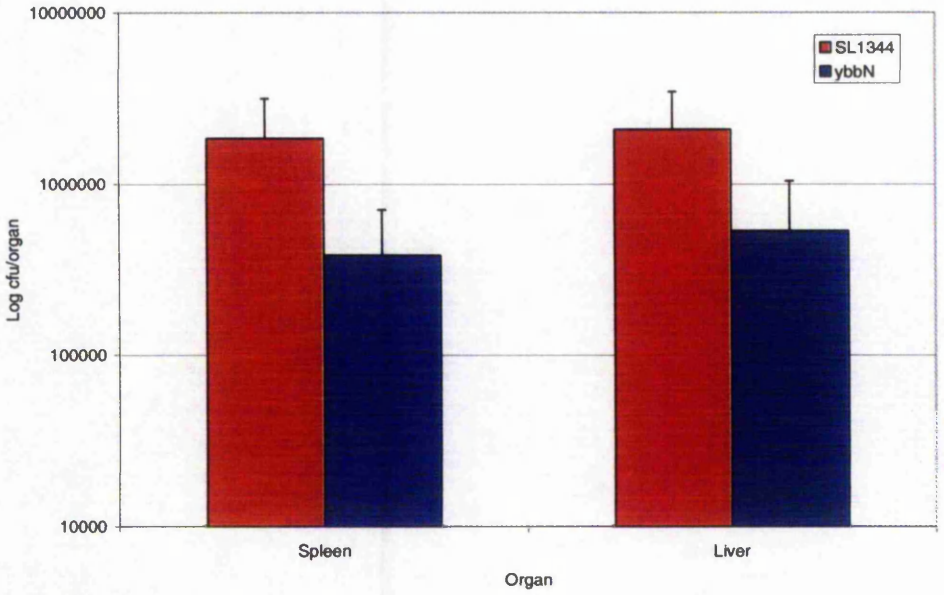


Figure 69. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*ybbN*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 0.25, and therefore Δ*ybbN* is just significantly different to the wild type strain.

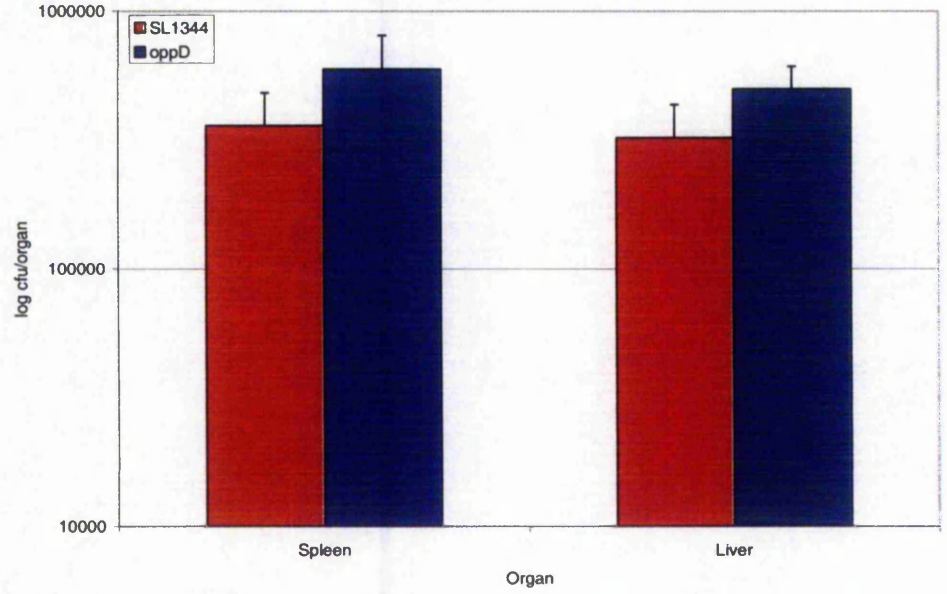


Figure 70. An IP competition assay of wild type *S.* Typhimurium SL1344 v Δ*oppD*::kan.

Data illustrated here is an average CFU/organ from 3 BALB/c mice. The error bars represent the standard deviation from the mean. This data equates to a CI of 1.1, and therefore Δ*oppD* is not significantly different to the wild type strain.

10.5 Discussion

The fourteen genes discussed in this chapter are interesting in many ways, particularly in that they stimulate more questions about the σ^E regulon of *S.* Typhimurium than they answer. We are now aware that two out of these fourteen genes (*mlc* and *yccV*) are only indirectly σ^E regulated. Inactivation of eleven of the remaining twelve genes confers none of the phenotypes associated with the *S.* Typhimurium *rpoE* mutant, other than that eight of them have some degree of growth defect at 46°C. The exception to this is *ybbN* which encodes for a thioredoxin like protein. This mutant is both temperature sensitive and significantly attenuated in BALB/c mice in comparison to the WT, SL1344 parent strain after IP administration. Thioredoxin is an electron donor involved in the reducing reaction of ribonucloetides to deoxyribonucleotides (Holmgren, 1985;Gleason and Holmgren, 1988), and shares a degree of functional redundancy in this process with glutathione (Holmgren, 1989). Thioredoxin can be reduced by a thioredoxin reductase (Williams, Jr., 1995). In the reduced state thioredoxin regulates the activities of enzymes in many cells by reducing disulfides to thiols (Aslund and Beckwith, 1999;Carmel-Harel and Storz, 2000;Williams *et al.*, 2000), and is also capable of affecting gene expression by binding to DNA gyrase (Li *et al.*, 2004). All of these are possible reasons for regulation by σ^E and also for the phenotype seen.

The reasons for the lack of phenotypes seen with the other genes could be numerous, including the possibility that there is enough functional overlap within the regulon to facilitate loss of some of the gene products, or that they are involved in pathways and functions that have as yet not been associated with regulation by σ^E, and we have not stressed/tested them appropriately. Indeed a combination of these suggestions could be true. The lack of attenuation observed with these mutants is also interesting. We

assumed when carrying out these experiments that we were the first group to look at the involvement of specific mutants of these genes in *S.* Typhimurium pathogenesis within a murine model or indeed any infection model. This was due to the absence of information from any virulence studies in the literature. However on reflection this may not be the case, and the absence of any reference to these genes and virulence may rather be due to the negative results which have not been published.

Of course this is not the end of the road for characterisation of these mutants, but merely the beginning. The aim now is to identify the role that these genes do play within the life cycle of *S.* Typhimurium, and test them under more conditions such as cold shock, now known to be one of the strongest inducers of σ^E (Kormanec J, Pers.Comm.) If this can be elucidated then the next step may be to construct double mutants and then look for phenotype to see if the functional overlap theory is correct. The single mutants could also be assessed in mice via different routes of infection and indeed in other infection models, as it is becoming more apparent that certain genes are required for *in vivo* survival depending on the host they are infecting (Morgan *et al.*, 2004).

Chapter 11- General Discussion

As each chapter has been discussed individually this general discussion emphasizes the major outcomes of the research and provides hypotheses and suggestions for future studies.

Typhoid fever is a severe, systemic infection caused by *Salmonella enterica* subspecies I serotype Typhi (*S.* Typhi). It is estimated that worldwide there are ~ 17 million cases of typhoid annually and that ~ 600, 000 people die of the infection worldwide (Pang *et al.*, 1998). Most of these infections occur in developing countries. In a recent outbreak of typhoid in Haiti, 200 cases of the disease and 40 deaths were reported (http://www.who.int/csr/don/2003_06_17a/en/). Antibiotics can be effective in the treatment of typhoid fever, but multiple antibiotic resistance is a major problem in certain areas of the world. There is a need for new antibacterial agents to treat typhoid fever and for more efficacious typhoid vaccines that can be used in all age groups. *S.* Typhi is a strictly human pathogen so with effective vaccines it would be possible to consider planning its global eradication. Infection of mice with *S.* Typhimurium causes a typhoid–like systemic infection (Baumler *et al.*, 1998) and because there is no suitable small animal model for *S.* Typhi, murine *S.* Typhimurium infection has been used as a model for systemic *Salmonella* infection. Using this model a number of virulence determinants have been identified that are essential for establishing and maintaining murine typhoid infection (Chatfield *et al.*, 1991;Johnson *et al.*, 1991;Miller, 1991;Chatfield *et al.*, 1992c;Baumler *et al.*, 1994;Jones and Falkow, 1996;Shea *et al.*, 1996;Yu and Kroll, 1999;Shea, Santangelo, and Feldman, 2000). The virulence capabilities of the regulatory genes of the ESR have been measured using this model, and inactivation of *rpoE,* the gene that encodes for the alternative sigma factor σ^E, in *S.* Typhimurium is severely attenuating (Humphreys *et al.*, 1999) whilst mutations in *cpxA* and *cpxR*, the genes that encode for the two component regulator which regulates the

other arm of the ESR, have a much smaller effect on *S.*Typhimurium virulence in the same model system. (Humphreys *et al.*, 2004). (Dartigalongue, Missiakas, and Raina, 2001) proposed that the *E. coli* σ^E regulon consists of approximately 43 genes; whilst (Rezuchova *et al.*, 2003) identified a further 11 σ^E regulated promoters in *E. coli* directing the expression of 15 genes. However, complete genomic sequencing of the enteric bacteria *E. coli* K12 and *S.* Typhimurium revealed that *E. coli* has around 800 genes absent from *S.* Typhimurium and that around 1100 *Salmonella* genes are absent from *E. coli* (McClelland and Wilson, 1998;McClelland *et al.*, 2001;Parkhill *et al.*, 2001). With this in mind and with the difference in the viability and other phenotypes of *rpoE* mutants between *S.* Typhimurium and *E. coli* (De Las, Connolly, and Gross, 1997a); the fact that *S.* Typhimurium is an intracellular pathogen, and the differences in non-host lifestyles (Winfield and Groisman, 2003), we hypothesized that there may be differential regulon members between these organisms. Recently phenotypic differences between *Salmonella* and *E. coli* resulting from disparate regulation of homologous genes have been identified (Winfield and Groisman, 2004), where the PmrA/PmrB systems respond differently to low magnesium concentrations. This evidence along with the differential regulation of RpoE between *E. coli* and *S.* Typhimurium which we have identified (Miticka *et al.*, 2003) adds weight to the argument that even though homologous systems have been characterized within *E. coli*, they should be investigated in their own right within related organisms. The principle aim of this thesis was to identify σ^E regulated genes in *S.* Typhimurium and then assess their importance for the ability of *S.* Typhimurium to cause disease using the murine typhoid model.

Using a tri-pronged approach we have identified 59 putative σ^E regulated genes in *S.* Typhimurium, of which a number have now been validated by S1 mapping and *lacZ* transcriptional fusions. One arm of our approach was to use a global genomic method,

as 2D electrophoresis had been fairly successful in *E. coli* in identifying σ^E regulated genes (Dartigalongue, Missiakas, and Raina, 2001). We opted to look at the effects of σ^E at the transcriptional level, using microarray analysis, as we predict this is where the most important role for σ^E, as an alternative sigma factor, would be. However it would be interesting to compare and contrast results obtained from the microarrays to 2D electrophoresis in *S.* Typhimurium as any differences between the two techniques may be as a result of a role for σ^E in post-transcriptional regulation, something that has not been considered thus far. This is plausible as σ^E may regulate genes involved in post-transcriptional regulation. The majority of the genes identified in *S.* Typhimurium as being σ^E regulated have a function related to the cell envelope, which is unsurprising as the role of σ^E is to regulate this environment. However a number of genes were also of unknown.

After using microarray analysis to investigate σ^E regulation it was particularly interesting to observe the potential indirect results of overexpression of RpoE. We were aware that due to the approach being used we would be activating the σ^H regulon but even so this does not account for the global effects seen. Future work should investigate the interactions between σ^E and other regulatory pathways of *Salmonella*, possibly by combining other regulatory mutations with the *rpoE* mutation and looking at the affects on global transcription. In this thesis we have performed some analyses of the interactions between σ^E and the Cpx regulated pathway, which indicates some degree of cross-regulation at the transcriptional level. However, this should be extended to other pathways outwith the ESR and in general the global effects caused by activation of σ^E should be considered rather than focusing too closely on one given pathway.

As well as trying to identify new members of the *S.* Typhimurium σ^E regulon we were keen to see if the signalling events that lead to σ^E activity in *E.* coli were also present in *S.* Typhimurium. Activation of σ^E in E. *coli* has been an area of popular research in recent months. In *E. coli* it is believed that DegS is an essential protein in this process (Alba *et al.*, 2001), and therefore itself essential for cell viability. The protease activity of DegS is activated through its binding of a C terminal peptide motif of misfolded outer membrane proteins (Walsh *et al.*, 2003). Once activated DegS is capable of the primary cleavage event required for dissociation of σ^E from its anti-sigma factor, RseA, which then allows a second protease, YaeL, to perform what was until recently thought to be the final cleavage event which allows free σ^E to bind to the core RNAP (Alba *et al.*, 2002). We have shown in this thesis and (Rowley *et al.*, 2005) that DegS is a non-essential protein in *S.* Typhimurium for activation of the σ^E pathway, although activity of this pathway is much greater in its presence. This then provokes the question as to how in *S.* Typhimurium σ^E is activated in the absence of DegS, which also provides stress sensing properties through the binding of its PDZ domain to misfiled OMPs within the periplasm. Can this activation be completed solely by YaeL? YaeL, now renamed RseP (regulon of sigma E, protease) (Akiyama, Kanehara, and Ito, 2004) is a bacterial orthologue of the eukaryotic S2P protease (Kanehara, Akiyama, and Ito, 2001). Until recently its only function in *E.* coli was thought to be the secondary site 2 cleavage of σ^E-RseA post primary cleavage by DegS (Kanehara, Ito, and Akiyama, 2002). Recently (Akiyama, Kanehara, and Ito, 2004) have demonstrated that the protease activity of RseP is capable of cleaving a number of substrates. This is in agreement with our inability to mutate this gene in *S.* Typhimurium, if it's sole function was to activate σ^E then a mutation of this gene should be feasible. In *Salmonella* we should construct truncated mutants of RseP, similar to those constructed in *E. coli*, and

look at the activity of σ^E in these mutants and also when combined with the *degS* mutation. In *E. coli* the absence of the PDZ domain of YaeL did not affect cell viability, and the proteolytic activity against RseA became independent of DegS (Bohn, Collier, and Bouloc, 2004), again implicating another method of sensing periplasmic stress and activating the σ^E response. The role of RseB in sensing periplasmic stress and activating the σ^E pathway should also be considered in *S.* Typhimurium. In *E. coli* a model was proposed whereby the RseB-RseA interaction represents a method of measuring misfolded polypeptides in the bacterial envelope, and enhancing the activity of RseA, thus modulating the assembly of σ^E RNA polymerase and the resulting cellular response (Collinet *et al.*, ;Missiakas *et al.*, 1997;De Las, Connolly, and Gross, 1997b). This work was somewhat superceded by the identification of the signaling pathway involving DegS and RseP, but this RseB model could be the result of the low level activation of σ^E observed in the absence of DegS, and could easily be analyzed by both combining the *degS* and *rseB* mutations and also by over expressing RseB in the absence of DegS.

After completion of the crystal structure of a σ^E-RseA complex it is now apparent that just the N terminal 90 amino acids of RseA is sufficient to inhibit σ^E, with a stoichiometric ratio of 1:1 (Campbell *et al.*, 2003). Therefore even after the sequential events described the σ^E-RseA complex that remains is still enough to prevent σ^E from binding to the polymerase. To allow binding. another cleavage event has been proposed involving the AAA+ protease ClpXP and an adaptor protein SspB (Flynn *et al.*, 2004). ClpXP performs a diverse array of cellular tasks including degradation of incomplete polypeptides and altering the response of regulatory proteins in response to stress (Gottesman, 1996;Schweder *et al.*, 1996;Gottesman *et al.*, 1998;Jones, Welty, and Nakai, 1998). The cellular function of ClpXP is further determined by adaptor proteins

which can fine tune ClpXP activity. SspB is such an adaptor protein, which was originally identified through its ability to enhance ClpXP degradation of SsrA tagged proteins (Dougan, Weber-Ban, and Bukau, 2003). (Flynn *et al.*, 2004) have now shown that a ClpXP-SspB complex is capable of cleaving σ^E from the remaining RseA fragment, allowing σ^E to bind to the core RNAP. However, the flaw in this argument is that both ClpX and ClpP are non essential for cell viability in *E. coli* K12, so either other proteases are capable of making this final cleavage or maybe σ^E is not essential in *E. coli* after all. We hypothesize that a number of proteins with protease activity are capable of providing this cleavage event. Sole reliance on proteolysis by ClpXP would not make evolutionary sense for the activation of such an important pathway. The activity of σ^E should be investigated in *S.* Typhimurium in the absence of ClpXP and the search for potential proteases that are also capable of providing this final cleavage event should be performed.

A continuation of the characterization of the σ^E regulon of *S.* Typhimurium performed in this thesis should be pursued along a number of avenues. Firstly, the remainder of the putative σ^E regulated genes should be confirmed by S1 mapping and *lacZ* transcriptional fusions, the structural CDS of these genes deleted, and characterization similar to that carried out in this thesis performed.

Secondly, those mutants identified in this study with a significant degree of attenuation, such as *tolR*, *skp* and *stm1250* should be investigated further. Complementation studies need to be performed where appropriate to confirm that the considered mutation is directly responsible for the phenotype seen, but complete complementation is not always feasible *in vivo* (Halsey *et al.*, 2004). Once this has been performed the murine immune response to these attenuated strains should be dissected, and they should also

be trialled as carriers of foreign antigen, and compared with the ability of the σ^E regulated gene, *htrA* in this role.

The role of σ^E regulated peptidyl-prolyl isomerases in *S.* Typhimurium virulence is something of great interest to us, and our ability to construct strains with combined mutants of these genes where this has proven non-viable in *E. coli*, again raises questions about the differences between the regulons of these organisms. Most of these isomerases also have chaperone function and the importance of the chaperone versus isomerase function *in vivo* should be dissected.

The mutants in this study that demonstrate notable, although not significant differences, in their virulence attributes should also be pursued further for two reasons. The animal group sizes used in the study was kept to an absolute minimum due to the number of mutants we wished to process, which may have an affect on the statistical significance. Such mutants where substantial differences from the wild type strain are observed after IP competition assay should either be repeated in larger groups by this same method or analyzed via the oral route. In mutants where no visible phenotype was observed *in vivo* they should be used to construct strains carrying multiple mutations, which should target multiple pathways. When *degQ*, the gene homologous to *htrA* and *degS*, is mutated no attenuation is seen, but when combined with the *htrA* mutation the overall attenuation is more significant than that of *htrA* alone (Farn and Roberts, 2004).

The ability of *S.* Typhimurium to infect a variety of hosts and environments, and the requirement for differential gene expression within these environments, presumably indicates a degree of gene redundancy depending on the current stage of the bacterium's life cycle. This is something else we need to bear in mind when we investigate the virulence properties of σ^E regulated genes in *S.* Typhimurium. Are certain σ^E regulated

genes important in the virulence process depending on the host? This should be investigated by analyzing some of the σ^E regulated genes in other model systems such as the calf and chick, to see if σ^E regulated genes that are attenuated in the murine model, are attenuated in these models. The *S.* Typhimurium *htrA* mutant is not attenuated in a calf model of infection (Villarreal-Ramos *et al.*, 2000). We should also investigate whether mutants not attenuated in the murine model become attenuated in these hosts.

We have now formulated an idea of the size of the σ^E regulon in *S.* Typhimurium and confirmed that this regulon does differ between that proposed for *E. coli* as expected. The concept of *Salmonella* specific genes which are under the control of RpoE is not something we had previously addressed and the evolution of this regulation is something that should be investigated further. With this in mind we are now keen to adapt our approach used here to identify RpoE regulated genes in other organisms, including other *Salmonella* species. Although the *S.* Typhimurium murine model has been very useful there are many differences between *S.* Typhi and *S.* Typhimurium and not all facet's of *S.* Typhi pathogenesis are likely to be gleaned from the study of *S.* Typhimurium. For example, *S.* Typhi produces a capsule (the Vi capsule or antigen) but *S.* Typhimurium does not. Also *S.* Typhi and *S.* Typhimurium strains with mutations in the same genes behave differently in human volunteers (Hindle *et al.*, 2002). Comparison of the genome sequences of *S.* Typhimurium LT2 and *S.* Typhi CT18 reveals that *S.* Typhi has 601 genes not present in *S.* Typhimurium and *S.* Typhimurium has 479 genes that are not present in *S.* Typhi (Parkhill *et al.*, 2001) (McClelland *et al.*, 2001) . Also *S.* Typhi has 204 pseudogenes, much higher than that predicted for *S.* Typhimurium (McClelland *et al.*, 2001) (Parkhill *et al.*, 2001). A number of *S.* Typhi pseudogenes are known or hypothesized to be involved in virulence or transmission in

S. Typhimurium (Parkhill *et al.*, 2001). Thus, there are pathogenic and genetic differences between *S.* Typhi and *S.* Typhimurium which justify studying the σ^E regulon of *S.* Typhi in its own right. Does the host specificity of *S.* Typhi alter the requirement for σ^E regulation of some of the genes identified in *S.* Typhimurium?

This thesis has focused on one arm of the ESR. Similar studies to those performed here should be undertaken to investigate the Cpx regulon of *S.* Typhimurium to again try and decipher any differences between *E. coli* and *S.* Typhimurium but more importantly in this case to ascertain which genes are co-regulated by both σ^E and Cpx. One would presume that the products of genes which have multiple levels of activation and regulation would play an important role within the bacterial life cycle, and possibly *in vivo*. Thus far the only genes known to be co-regulated by both Cpx and σ^E are *htrA* and *skp*, mutations of which are both important in *S.* Typhimurium infection of mice. Along the same vein of reasoning a search should be made for genes which are co-regulated by both σ^E and σ^H. We are the first group to identify a gene which is co-regulated by both of these alternative sigma factors, the *Salmonella* specific gene *stm1251*.

The importance of σ^E for survival in *E. coli* K12 but not in a number of other closely related bacteria is an interesting phenomenon. What is the essential role that σ^E plays in *E. coli* K12 and how in evolutionary terms did an alternative sigma factor become an essential sigma factor? Does K12 possess a secondary mutation that renders a mutation in σ^E non-viable? These are questions that are never addressed. It was originally proposed that a mutation in σ^E of *S.* Typhimurium is only possible due to a secondary suppressor mutation, but this is unlikely as σ^E is being disrupted in more and more species of bacteria. It would therefore be wise for someone to try and mutate σ^E in wild type pathogenic strains of *E. coli* rather than a laboratory passaged strain.

In conclusion, this thesis has begun to shape the σ^E regulon of *S.* Typhimurium It has further elucidated the importance of σ^E regulated genes in the virulence process of *S.* Typhimurium and has indicated the degree of complexity involved in the activation and regulation of such a system and in its interactions with similar regulatory systems.

Bibliography

Aasland,R., Coleman,J., Holck,A.L., Smith,C.L., Raetz,C.R., and Kleppe,K. (1988) Identity of the 17-kilodalton protein, a DNA-binding protein from Escherichia coli, and the firA gene product *J.Bacteriol.* **170**: 5916-5918.

Abe,A., Matsui,H., Danbara,H., Tanaka,K., Takahashi,H., and Kawahara,K. (1994) Regulation of spvR gene expression of Salmonella virulence plasmid pKDSC50 in Salmonella choleraesuis serovar Choleraesuis *Mol.Microbiol.* **12**: 779-787.

Acharya,I.L., Lowe,C.U., Thapa,R., Gurubacharya,V.L., Shrestha,M.B., Cadoz,M., Schulz,D., Armand,J., Bryla,D.A., Trollfors,B., and . (1987) Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of Salmonella typhi. A preliminary report *N.Engl.J.Med.* **317**: 1101-1104.

Ades,S.E. (2004) Control of the alternative sigma factor sigmaE in Escherichia coli *Curr.Opin.Microbiol.* **7**: 157-162.

Ades,S.E., Connolly,L.E., Alba,B.M., and Gross,C.A. (1999) The Escherichia coli sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor *Genes Dev.* **13**: 2449-2461.

Akiyama,Y., Kanehara,K., and Ito,K. (2004) RseP (YaeL), an Escherichia coli RIP protease, cleaves transmembrane sequences *EMBO J.* **23**: 4434-4442.

Alba,B.M., Gross,C.A. (2004) Regulation of the Escherichia colisigma-dependent envelope stress response *Mol.Microbiol.* **52**: 613-619.

Alba,B.M., Leeds,J.A., Onufryk,C., Lu,C.Z., and Gross,C.A. (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response *Genes Dev.* **16**: 2156-2168.

Alba,B.M., Zhong,H.J., Pelayo,J.C., and Gross,C.A. (2001) degS (hhoB) is an essential Escherichia coli gene whose indispensable function is to provide sigma activity *Mol.Microbiol.* **40**: 1323-1333.

Albin,R., Weber,R., and Silverman,P.M. (1986) The Cpx proteins of Escherichia coli K12. Immunologic detection of the chromosomal cpxA gene product *J Biol.Chem.* **261**: 4698-4705.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W., and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs *Nucleic Acids Res.* **25**: 3389-3402.

Anderson,E.S. (1968) Drug resistance in Salmonella typhimurium and its implications *Br.Med.J.* **3**: 333-339.

Andrews,J.C., Blevins,T.C., and Short,S.A. (1986) Regulation of peptide transport in Escherichia coli: induction of the trp-linked operon encoding the oligopeptide permease *J.Bacteriol.* **165**: 428-433.

Andrews,J.C., Short,S.A. (1985) Genetic analysis of Escherichia coli oligopeptide transport mutants *J.Bacteriol.* **161**: 484-492.

Archambault,J., Friesen,J.D. (1993) Genetics of eukaryotic RNA polymerases I, II, and III *Microbiol.Rev.* **57**: 703-724.

Aslund,F., Beckwith,J. (1999) The thioredoxin superfamily: redundancy, specificity, and gray-area genomics *J.Bacteriol.* **181**: 1375-1379.

Bader,M.W., Navarre,W.W., Shiau,W., Nikaido,H., Frye,J.G., McClelland,M., Fang,F.C., and Miller,S.I. (2003) Regulation of Salmonella typhimurium virulence gene expression by cationic antimicrobial peptides *Mol.Microbiol.* **50**: 219-230.

Bajaj,V., Hwang,C., and Lee,C.A. (1995) hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes *Mol.Microbiol.* **18**: 715-727.

Bajaj,V., Lucas,R.L., Hwang,C., and Lee,C.A. (1996) Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression *Mol.Microbiol.* **22**: 703-714.

Bakshi,C.S., Singh,V.P., Wood,M.W., Jones,P.W., Wallis,T.S., and Galyov,E.E. (2000) Identification of SopE2, a Salmonella secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells *J.Bacteriol.* **182**: 2341-2344.

Baranova,N., Nikaido,H. (2002) The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate *J.Bacteriol.* **184**: 4168-4176.

Barth,M., Marschall,C., Muffler,A., Fischer,D., and Hengge-Aronis,R. (1995) Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in Escherichia coli *J.Bacteriol.* **177**: 3455-3464.

Bass,S., Gu,Q., and Christen,A. (1996) Multicopy suppressors of prc mutant Escherichia coli include two HtrA (DegP) protease homologs (HhoAB), DksA, and a truncated R1pA *J.Bacteriol.* **178**: 1154-1161.

Bateman,A., Coin,L., Durbin,R., Finn,R.D., Hollich,V., Griffiths-Jones,S., Khanna,A., Marshall,M., Moxon,S., Sonnhammer,E.L., Studholme,D.J., Yeats,C., and Eddy,S.R. (2004) The Pfam protein families database *Nucleic Acids Res.* **32 Database issue:D138-41.**: D138-D141.

Baudin,A., Ozier-Kalogeropoulos,O., Denouel,A., Lacroute,F., and Cullin,C. (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae *Nucleic Acids Res.* **21**: 3329-3330.

Baumler,A.J., Kusters,J.G., Stojiljkovic,I., and Heffron,F. (1994) Salmonella typhimurium loci involved in survival within macrophages *Infect Immun.* **62**: 1623-1630.

Baumler,A.J., Tsolis,R.M., and Heffron,F. (1996a) Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by Salmonella typhimurium *Infect.Immun.* **64**: 1862-1865.

Baumler,A.J., Tsolis,R.M., and Heffron,F. (1996b) The lpf fimbrial operon mediates adhesion of Salmonella typhimurium to murine Peyer's patches *Proc.Natl.Acad.Sci.U.S.A* **93**: 279-283.

Baumler,A.J., Tsolis,R.M., and Heffron,F. (1997) Fimbrial adhesins of Salmonella typhimurium. Role in bacterial interactions with epithelial cells *Adv.Exp.Med.Biol.* **412**: 149-158.

Baumler,A.J., Tsolis,R.M., Ficht,T.A., and Adams,L.G. (1998) Evolution of host adaptation in Salmonella enterica *Infect Immun* **66**: 4579-4587.

Behlau,I., Miller,S.I. (1993) A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells *J.Bacteriol.* **175**: 4475-4484.

Bergler,H., Abraham,D., Aschauer,H., and Turnowsky,F. (1994) Inhibition of lipid biosynthesis induces the expression of the pspA gene *Microbiology* **140**: 1937-1944.

Bernstein,A., Rolfe,B., and Onodera,K. (1972) Pleiotropic properties and genetic organization of the tolA,B locus of Escherichia coli K-12 *J.Bacteriol.* **112**: 74-83.

Beuzon,C.R., Holden,D.W. (2001) Use of mixed infections with Salmonella strains to study virulence genes and their interactions in vivo *Microbes.Infect.* **3**: 1345-1352.

Bhat,U.R., Carlson,R.W., Busch,M., and Mayer,H. (1991a) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria *Int.J.Syst.Bacteriol.* **41**: 213-217.

Bhat,U.R., Mayer,H., Yokota,A., Hollingsworth,R.I., and Carlson,R.W. (1991b) Occurrence of lipid A variants with 27-hydroxyoctacosanoic acid in lipopolysaccharides from members of the family Rhizobiaceae *J.Bacteriol.* **173**: 2155-2159.

Blanc-Potard,A.B., Groisman,E.A. (1997) The Salmonella selC locus contains a pathogenicity island mediating intramacrophage survival *EMBO J.* **16**: 5376-5385.

Blanc-Potard,A.B., Solomon,F., Kayser,J., and Groisman,E.A. (1999) The SPI-3 pathogenicity island of Salmonella enterica *J.Bacteriol.* **181**: 998-1004.

Bohn,C., Collier,J., and Bouloc,P. (2004) Dispensable PDZ domain of Escherichia coli YaeL essential protease *Mol.Microbiol.* **52**: 427-435.

Borukhov,S., Nudler,E. (2003) RNA polymerase holoenzyme: structure, function and biological implications *Curr.Opin.Microbiol.* **6**: 93-100.

Bosch,D., Tommassen,J. (1987) Effects of linker insertions on the biogenesis and functioning of the Escherichia coli outer membrane pore protein PhoE *Mol.Gen.Genet.* **208**: 485-489.

Bourdet-Sicard,R., Tran,V.N. (1999) Actin reorganization by SipA and Salmonella invasion of epithelial cells *Trends Microbiol.* **7**: 309-310.

Bouveret,E., Derouiche,R., Rigal,A., Lloubes,R., Lazdunski,C., and Benedetti,H. (1995) Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of Escherichia coli *J.Biol.Chem.* **270**: 11071-11077.

Boyd,E.F., Wang,F.S., Whittam,T.S., and Selander,R.K. (1996) Molecular genetic relationships of the salmonellae *Appl.Environ.Microbiol.* **62**: 804-808.

Braun,V., Herrmann,C. (1993) Evolutionary relationship of uptake systems for biopolymers in Escherichia coli: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins *Mol.Microbiol.* **8**: 261-268.

Brissette,J.L., Russel,M., Weiner,L., and Model,P. (1990) Phage shock protein, a stress protein of Escherichia coli *Proc.Natl.Acad.Sci.U.S.A* **87**: 862-866.

Brissette,J.L., Weiner,L., Ripmaster,T.L., and Model,P. (1991) Characterization and sequence of the Escherichia coli stress-induced psp operon *J.Mol.Biol.* **220**: 35-48.

British National Formulary, 2004. British Medical Association and Royal Pharmaceutical Society of Great Britain. 48. 2004.
Ref Type: Generic

Brown,L., Elliott,T. (1996) Efficient translation of the RpoS sigma factor in Salmonella typhimurium requires host factor I, an RNA-binding protein encoded by the hfq gene *J.Bacteriol.* **178**: 3763-3770.

Buchmeier,N.A., Lipps,C.J., So,M.Y., and Heffron,F. (1993) Recombination-deficient mutants of Salmonella typhimurium are avirulent and sensitive to the oxidative burst of macrophages *Mol.Microbiol.* **7**: 933-936.

Bukau,B. (1993) Regulation of the Escherichia coli heat-shock response *Mol.Microbiol.* **9**: 671-680.

Bulling,E., Stephan,R., and Sebek,V. (1973) [The development of antibiotics resistance among salmonella bacteria of animal origin in the Federal Republic of Germany and West Berlin. 1st communication: a comparison between the years of 1961 and 1970-71 (author's transl)] *Zentralbl.Bakteriol.[Orig.A]* **225**: 245-256.

Campbell,E.A., Tupy,J.L., Gruber,T.M., Wang,S., Sharp,M.M., Gross,C.A., and Darst,S.A. (2003) Crystal structure of Escherichia coli sigmaE with the cytoplasmic domain of its anti-sigma RseA *Mol.Cell* **11**: 1067-1078.

Canh,d.G., Lin,F.Y., Thiem,V.D., Trach,D.D., Trong,N.D., Mao,N.D., Hunt,S., Schneerson,R., Robbins,J.B., Chu,C., Shiloach,J., Bryla,D.A., Bonnet,M.C., Schulz,D., and Szu,S.C. (2004) Effect of dosage on immunogenicity of a Vi conjugate vaccine injected twice into 2- to 5-year-old Vietnamese children *Infect Immun.* **72**: 6586-6588.

Cannon,W., Claverie-Martin,F., Austin,S., and Buck,M. (1994) Identification of a DNA-contacting surface in the transcription factor sigma-54 *Mol.Microbiol.* **11**: 227-236.

Cannon,W., Missailidis,S., Smith,C., Cottier,A., Austin,S., Moore,M., and Buck,M. (1995) Core RNA polymerase and promoter DNA interactions of purified domains of sigma N: bipartite functions *J.Mol.Biol.* **248**: 781-803.

Canonne-Hergaux,F., Gruenheid,S., Govoni,G., and Gros,P. (1999) The Nramp1 protein and its role in resistance to infection and macrophage function *Proc.Assoc.Am.Physicians* **111**: 283-289.

Carmel-Harel,O., Storz,G. (2000) Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress *Annu.Rev.Microbiol.* **54:439-61.**: 439-461.

Carter,P.B., Collins,F.M. (1974) The route of enteric infection in normal mice *J.Exp.Med.* **139**: 1189-1203.

Cascales,E., Gavioli,M., Sturgis,J.N., and Lloubes,R. (2000) Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in Escherichia coli *Mol.Microbiol.* **38**: 904-915.

Cascales,E., Lloubes,R., and Sturgis,J.N. (2001) The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB *Mol.Microbiol.* **42**: 795-807.

Chalker,A.F., Ingraham,K.A., Lunsford,R.D., Bryant,A.P., Bryant,J., Wallis,N.G., Broskey,J.P., Pearson,S.C., and Holmes,D.J. (2000) The bacA gene, which determines bacitracin susceptibility in Streptococcus pneumoniae and Staphylococcus aureus, is also required for virulence *Microbiology* **146**: 1547-1553.

Chan,K., Baker,S., Kim,C.C., Detweiler,C.S., Dougan,G., and Falkow,S. (2003) Genomic comparison of Salmonella enterica serovars and Salmonella bongori by use of an S. enterica serovar typhimurium DNA microarray *J.Bacteriol.* **185**: 553-563.

Chang,Y.Y., Cronan,J.E., Jr. (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of Escherichia coli *Mol.Microbiol.* **33**: 249-259.

Chatfield,S.N., Charles,I.G., Makoff,A.J., Oxer,M.D., Dougan,G., Pickard,D., Slater,D., and Fairweather,N.F. (1992a) Use of the nirB promoter to direct the stable expression of heterologous antigens in Salmonella oral vaccine strains: development of a single-dose oral tetanus vaccine *Biotechnology (N.Y.)* **10**: 888-892.

Chatfield,S.N., Dorman,C.J., Hayward,C., and Dougan,G. (1991) Role of ompR-dependent genes in Salmonella typhimurium virulence: mutants deficient in both ompC and ompF are attenuated in vivo *Infect Immun.* **59**: 449-452.

Chatfield,S.N., Fairweather,N., Charles,I., Pickard,D., Levine,M., Hone,D., Posada,M., Strugnell,R.A., and Dougan,G. (1992b) Construction of a genetically defined Salmonella typhi Ty2 aroA, aroC mutant for the engineering of a candidate oral typhoid-tetanus vaccine *Vaccine* **10**: 53-60.

Chatfield,S.N., Strahan,K., Pickard,D., Charles,I.G., Hormaeche,C.E., and Dougan,G. (1992c) Evaluation of Salmonella typhimurium strains harbouring defined mutations in htrA and aroA in the murine salmonellosis model *Microb.Pathog.* **12**: 145-151.

Chaudhuri,R.R., Khan,A.M., and Pallen,M.J. (2004) coliBASE: an online database for Escherichia coli, Shigella and Salmonella comparative genomics *Nucleic Acids Res.* **32 Database issue**: D296-D299.

Chen,C.Y., Buchmeier,N.A., Libby,S., Fang,F.C., Krause,M., and Guiney,D.G. (1995) Central regulatory role for the RpoS sigma factor in expression of Salmonella dublin plasmid virulence genes *J.Bacteriol.* **177**: 5303-5309.

Chen,R., Henning,U. (1996) A periplasmic protein (Skp) of Escherichia coli selectively binds a class of outer membrane proteins *Mol.Microbiol.* **19**: 1287-1294.

Cherepanov,P.P., Wackernagel,W. (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant *Gene* **158**: 9-14.

Cirillo,D.M., Valdivia,R.H., Monack,D.M., and Falkow,S. (1998) Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival *Mol.Microbiol.* **30**: 175-188.

Clark,M.A., Hirst,B.H., and Jepson,M.A. (1998) Inoculum composition and Salmonella pathogenicity island 1 regulate M-cell invasion and epithelial destruction by Salmonella typhimurium *Infect.Immun.* **66**: 724-731.

Clavel,T., Lazzaroni,J.C., Vianney,A., and Portalier,R. (1996) Expression of the tolQRA genes of Escherichia coli K-12 is controlled by the RcsC sensor protein involved in capsule synthesis *Mol.Microbiol.* **19**: 19-25.

Clements,M.O., Eriksson,S., Thompson,A., Lucchini,S., Hinton,J.C., Normark,S., and Rhen,M. (2002) Polynucleotide phosphorylase is a global regulator of virulence and persistency in Salmonella enterica *Proc.Natl.Acad.Sci.U.S.A* **99**: 8784-8789.

Collazo,C.M., Galan,J.E. (1997) The invasion-associated type-III protein secretion system in Salmonella--a review *Gene* **192**: 51-59.

Collinet,B., Yuzawa,H., Chen,T., Herrera,C., and Missiakas,D. RseB binding to the periplasmic domain of RseA modulates the RseA:sigmaE interaction in the cytoplasm and the availability of sigmaE.RNA polymerase *J.Biol.Chem.2000.Oct.27.;275.(43.):33898.-904.* **275**: 33898-33904.

Connolly,L., De Las,P., Alba,B.M., and Gross,C.A. (1997) The response to extracytoplasmic stress in Escherichia coli is controlled by partially overlapping pathways *Genes Dev.* **11**: 2012-2021.

Cosloy,S.D., Oishi,M. (1973a) Genetic transformation in Escherichia coli K12 *Proc.Natl.Acad.Sci.U.S.A* **70**: 84-87.

Cosloy,S.D., Oishi,M. (1973b) The nature of the transformation process in Escherichia coli K12 *Mol.Gen.Genet.* **124**: 1-10.

Coyne,M.J., Jr., Russell,K.S., Coyle,C.L., and Goldberg,J.B. (1994) The Pseudomonas aeruginosa algC gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core *J.Bacteriol.* **176**: 3500-3507.

Craig,E.A., Gross,C.A. (1991) Is hsp70 the cellular thermometer? *Trends Biochem.Sci.* **16**: 135-140.

Craig,J.E., Nobbs,A., and High,N.J. (2002) The extracytoplasmic sigma factor, final sigma(E), is required for intracellular survival of nontypeable Haemophilus influenzae in J774 macrophages *Infect.Immun.* **70**: 708-715.

Crerar,S.K., Nicholls,T.J., and Barton,M.D. (1999) Multi-resistant Salmonella Typhimurium DT104--implications for animal industries and the veterinary profession *Aust.Vet.J.* **77**: 170-171.

Crosa,J.H., Brenner,D.J., Ewing,W.H., and Falkow,S. (1973) Molecular relationships among the Salmonelleae *J.Bacteriol.* **115**: 307-315.

Cryz,S.J., Furer,E. (1988) Further characterization of the Salmonella typhi Ty21a vaccine strain *J.Infect.Dis.* **157**: 1276-1279.

Cullen,P.J., Foster-Hartnett,D., Gabbert,K.K., and Kranz,R.G. (1994) Structure and expression of the alternative sigma factor, RpoN, in Rhodobacter capsulatus; physiological relevance of an autoactivated nifU2-rpoN superoperon *Mol.Microbiol.* **11**: 51-65.

Curtiss,R., III, Kelly,S.M., Gulig,P.A., and Nakayama,K. (1989a) Selective delivery of antigens by recombinant bacteria *Curr.Top.Microbiol.Immunol.* **146:35-49.**: 35-49.

Curtiss,R., III, Kelly,S.M., Gulig,P.A., and Nakayama,K. (1989b) Stable recombinant avirulent Salmonella vaccine strains *Adv.Exp.Med.Biol.* **251:33-47.**: 33-47.

d'Alencon,E., Taghbalout,A., Bristow,C., Kern,R., Aflalo,R., and Kohiyama,M. (2003) Isolation of a new hemimethylated DNA binding protein which regulates dnaA gene expression *J.Bacteriol.* **185**: 2967-2971.

Danese,P.N., Silhavy,T.J. (1998) CpxP, a stress-combative member of the Cpx regulon *J.Bacteriol.* **180**: 831-839.

Danese,P.N., Snyder,W.B., Cosma,C.L., Davis,L.J., and Silhavy,T.J. (1995) The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP *Genes Dev.* **9**: 387-398.

Dartigalongue,C., Loferer,H., and Raina,S. (2001) EcfE, a new essential inner membrane protease: its role in the regulation of heat shock response in Escherichia coli *EMBO J.* **20**: 5908-5918.

Dartigalongue,C., Missiakas,D., and Raina,S. (2001) Characterization of the Escherichia coli sigma E regulon *J.Biol.Chem.* **276**: 20866-20875.

Darwin,A.J., Miller,V.L. (2001) The psp locus of Yersinia enterocolitica is required for virulence and for growth in vitro when the Ysc type III secretion system is produced *Mol.Microbiol.* **39**: 429-444.

Darwin,K.H., Miller,V.L. (1999) InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium *J.Bacteriol.* **181**: 4949-4954.

Datsenko,K.A., Wanner,B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products *Proc.Natl.Acad.Sci.U.S.A* **97**: 6640-6645.

de Cock,H., Schafer,U., Potgeter,M., Demel,R., Muller,M., and Tommassen,J. (1999) Affinity of the periplasmic chaperone Skp of Escherichia coli for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Skp in the biogenesis of outer membrane protein *Eur.J.Biochem.* **259**: 96-103.

De Las,P., Connolly,L., and Gross,C.A. (1997a) SigmaE is an essential sigma factor in Escherichia coli *J Bacteriol.* **179**: 6862-6864.

De Las,P., Connolly,L., and Gross,C.A. (1997b) The sigmaE-mediated response to extracytoplasmic stress in Escherichia coli is transduced by RseA and RseB, two negative regulators of sigmaE *Mol.Microbiol.* **24**: 373-385.

De Wulf,P., Akerley,B.J., and Lin,E.C. (2000) Presence of the Cpx system in bacteria *Microbiology* **146**: 247-248.

De Wulf,P., Kwon,O., and Lin,E.C. (1999) The CpxRA signal transduction system of Escherichia coli: growth-related autoactivation and control of unanticipated target operons *J.Bacteriol.* **181**: 6772-6778.

De Wulf,P., Lin,E.C. (2000) Cpx two-component signal transduction in Escherichia coli: excessive CpxR-P levels underlie CpxA* phenotypes *J.Bacteriol.* **182**: 1423-1426.

De Wulf,P., McGuire,A.M., Liu,X., and Lin,E.C. (2002) Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in Escherichia coli *J.Biol.Chem.* **%19;277**: 26652-26661.

Dennis,J.J., Lafontaine,E.R., and Sokol,P.A. (1996) Identification and characterization of the tolQRA genes of Pseudomonas aeruginosa *J.Bacteriol.* **178**: 7059-7068.

Deretic,V., Dikshit,R., Konyecsni,W.M., Chakrabarty,A.M., and Misra,T.K. (1989) The algR gene, which regulates mucoidy in Pseudomonas aeruginosa, belongs to a class of environmentally responsive genes *J.Bacteriol.* **171**: 1278-1283.

Deretic,V., Konyecsni,W.M. (1989) Control of mucoidy in Pseudomonas aeruginosa: transcriptional regulation of algR and identification of the second regulatory gene, algQ *J.Bacteriol.* **171**: 3680-3688.

Deretic,V., Schurr,M.J., and Yu,H. (1995) Pseudomonas aeruginosa, mucoidy and the chronic infection phenotype in cystic fibrosis *Trends.Microbiol.* **3**: 351-356.

DeVries,C.A., Ohman,D.E. (1994) Mucoid-to-nonmucoid conversion in alginate-producing Pseudomonas aeruginosa often results from spontaneous mutations in algT, encoding a putative alternate sigma factor, and shows evidence for autoregulation *J.Bacteriol.* **176**: 6677-6687.

Dong,J., Iuchi,S., Kwan,H.S., Lu,Z., and Lin,E.C. (1993) The deduced amino-acid sequence of the cloned cpxR gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of Escherichia coli *Gene* **136**: 227-230.

Donnenberg,M.S., Kaper,J.B. (1991) Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector *Infect Immun.* **59**: 4310-4317.

Dougan,D.A., Weber-Ban,E., and Bukau,B. (2003) Targeted delivery of an ssrA-tagged substrate by the adaptor protein SspB to its cognate AAA+ protein ClpX *Mol.Cell* **12**: 373-380.

Dougan,G., Chatfield,S., Pickard,D., Bester,J., O'Callaghan,D., and Maskell,D. (1988) Construction and characterization of vaccine strains of Salmonella harboring mutations in two different aro genes *J Infect Dis* **158**: 1329-1335.

Drew,D., Sjostrand,D., Nilsson,J., Urbig,T., Chin,C.N., de Gier,J.W., and von Heijne,G. (2002) Rapid topology mapping of Escherichia coli inner-membrane proteins by prediction and PhoA/GFP fusion analysis *Proc.Natl.Acad.Sci.U.S.A* **99**: 2690-2695.

Edsall,G., Gaines,S., Landy,M., Tigertt,W.D., Sprinz,H., Trapani,R.J., Mandel,A.D., and Benenson,A.S. (1960) Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with Salmonella typhosa *J.Exp.Med.* **112**: 143-166.

Edwards,P.R., Kauffmann,F. (1952) A simplification of the Kauffmann-White schema *Am.J.Clin.Pathol.* **22**: 692-697.

Edwards,R.A., Olsen,G.J., and Maloy,S.R. (2002) Comparative genomics of closely related salmonellae *Trends Microbiol.* **10**: 94-99.

Eichelberg,K., Galan,J.E. (1999) Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA *Infect.Immun.* **67**: 4099-4105.

Eichelberg,K., Ginocchio,C.C., and Galan,J.E. (1994) Molecular and functional characterization of the Salmonella typhimurium invasion genes invB and invC: homology of InvC to the F0F1 ATPase family of proteins *J.Bacteriol.* **176**: 4501-4510.

Eick-Helmerich,K., Braun,V. (1989) Import of biopolymers into Escherichia coli: nucleotide sequences of the exbB and exbD genes are homologous to those of the tolQ and tolR genes, respectively *J.Bacteriol.* **171**: 5117-5126.

Eisenstein,T.K., Killar,L.M., and Sultzer,B.M. (1984) Immunity to infection with Salmonella typhimurium: mouse-strain differences in vaccine- and serum-mediated protection *J.Infect.Dis.* **150**: 425-435.

El Ghachi,M., Bouhss,A., Blanot,D., and Mengin-Lecreulx,D. (2004) The bacA gene of Escherichia coli encodes an undecaprenyl pyrophosphate phosphatase activity *J.Biol.Chem.* **279**: 30106-30113.

Ellermeier,C.D., Janakiraman,A., and Slauch,J.M. (2002) Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria *Gene* **290**: 153-161.

Erickson,J.W., Gross,C.A. (1989) Identification of the sigma E subunit of Escherichia coli RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression *Genes Dev.* **3**: 1462-1471.

Erickson,J.W., Vaughn,V., Walter,W.A., Neidhardt,F.C., and Gross,C.A. (1987) Regulation of the promoters and transcripts of rpoH, the Escherichia coli heat shock regulatory gene *Genes Dev.* **1**: 419-432.

Eriksson,S., Lucchini,S., Thompson,A., Rhen,M., and Hinton,J.C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica *Mol.Microbiol.* **47**: 103-118.

Ernst,R.K., Dombroski,D.M., and Merrick,J.M. (1990) Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by Salmonella typhimurium *Infect.Immun.* **58**: 2014-2016.

Espinosa-Urgel,M., Chamizo,C., and Tormo,A. (1996) A consensus structure for sigma S-dependent promoters *Mol.Microbiol.* **21**: 657-659.

Espinosa-Urgel,M., Tormo,A. (1993) Sigma s-dependent promoters in Escherichia coli are located in DNA regions with intrinsic curvature *Nucleic Acids Res.* **21**: 3667-3670.

Everest,P., Frankel,G., Li,J., Lund,P., Chatfield,S., and Dougan,G. (1995) Expression of LacZ from the htrA, nirB and groE promoters in a Salmonella vaccine strain: influence of growth in mammalian cells *FEMS Microbiol Lett.* **126**: 97-101.

Everest,P., Griffiths,P., and Dougan,G. (1995) Live Salmonella vaccines as a route towards oral immunisation *Biologicals* **23**: 119-124.

Everest,P., Wain,J., Roberts,M., Rook,G., and Dougan,G. (2001) The molecular mechanisms of severe typhoid fever *Trends Microbiol.* **9**: 316-320.

Fairweather,N.F., Chatfield,S.N., Makoff,A.J., Strugnell,R.A., Bester,J., Maskell,D.J., and Dougan,G. (1990) Oral vaccination of mice against tetanus by use of a live attenuated Salmonella carrier *Infect Immun.* **58**: 1323-1326.

Fang,F.C., Libby,S.J., Buchmeier,N.A., Loewen,P.C., Switala,J., Harwood,J., and Guiney,D.G. (1992) The alternative sigma factor katF (rpoS) regulates Salmonella virulence *Proc.Natl.Acad.Sci.U.S.A* **89**: 11978-11982.

Farn,J., Roberts,M. (2004) Effect of Inactivation of the HtrA-Like Serine Protease DegQ on the Virulence of Salmonella enterica Serovar Typhimurium in Mice *Infect.Immun.* **72**: 7357-7359.

Felix A and Pitt R.M. A new antigen of B. typhosus its relation to virulence and to active and passive imunization. Lancet 2, 186-191. 1934.
Ref Type: Generic

Ferguson,G.P., Roop,R.M., and Walker,G.C. (2002) Deficiency of a Sinorhizobium meliloti BacA mutant in alfalfa symbiosis correlates with alteration of the cell envelope *J.Bacteriol.* **184**: 5625-5632.

Finch,P.W., Wilson,R.E., Brown,K., Hickson,I.D., and Emmerson,P.T. (1986) Complete nucleotide sequence of the Escherichia coli ptr gene encoding protease III *Nucleic Acids Res.* **14**: 7695-7703.

Finlay,B.B., Brumell,J.H. (2000) Salmonella interactions with host cells: in vitro to in vivo *Philos.Trans.R.Soc.Lond B Biol.Sci.* **355**: 623-631.

Firoved,A.M., Boucher,J.C., and Deretic,V. (2002) Global genomic analysis of AlgU (sigma(E))-dependent promoters (sigmulon) in Pseudomonas aeruginosa and implications for inflammatory processes in cystic fibrosis *J.Bacteriol.* **184**: 1057-1064.

Firoved,A.M., Deretic,V. (2003) Microarray analysis of global gene expression in mucoid Pseudomonas aeruginosa *J.Bacteriol.* **185**: 1071-1081.

Fisher,S.L., Jiang,W., Wanner,B.L., and Walsh,C.T. (1995) Cross-talk between the histidine protein kinase VanS and the response regulator PhoB. Characterization and identification of a VanS domain that inhibits activation of PhoB *J.Biol.Chem.* **270**: 23143-23149.

Flynn,J.M., Levchenko,I., Sauer,R.T., and Baker,T.A. (2004) Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation *Genes Dev.* **18**: 2292-2301.

Fognini-Lefebvre,N., Lazzaroni,J.C., and Portalier,R. (1987) tolA, tolB and excC, three cistrons involved in the control of pleiotropic release of periplasmic proteins by Escherichia coli K12 *Mol.Gen.Genet.* **209**: 391-395.

Foster,J.W. (1991) Salmonella acid shock proteins are required for the adaptive acid tolerance response *J.Bacteriol.* **173**: 6896-6902.

Foster,J.W. (1993) The acid tolerance response of Salmonella typhimurium involves transient synthesis of key acid shock proteins *J.Bacteriol.* **175**: 1981-1987.

Foster,J.W., Moreno,M. (1999) Inducible acid tolerance mechanisms in enteric bacteria *Novartis.Found.Symp.* **221:55-69; discussion 70-4.**: 55-69.

Foster,J.W., Spector,M.P. (1995) How Salmonella survive against the odds *Annu.Rev.Microbiol* **49:145-74**: 145-174.

Freudenberg,M.A., Merlin,T., Gumenscheimer,M., Kalis,C., Landmann,R., and Galanos,C. (2001) Role of lipopolysaccharide susceptibility in the innate immune response to Salmonella typhimurium infection: LPS, a primary target for recognition of Gram-negative bacteria *Microbes.Infect.* **3**: 1213-1222.

Friebel,A., Ilchmann,H., Aepfelbacher,M., Ehrbar,K., Machleidt,W., and Hardt,W.D. (2001) SopE and SopE2 from Salmonella typhimurium activate different sets of RhoGTPases of the host cell *J.Biol.Chem.* **276**: 34035-34040.

Fu,Y., Galan,J.E. (1998a) Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of Salmonella typhimurium *J.Bacteriol.* **180**: 3393-3399.

Fu,Y., Galan,J.E. (1998b) The Salmonella typhimurium tyrosine phosphatase SptP is translocated into host cells and disrupts the actin cytoskeleton *Mol.Microbiol.* **27**: 359-368.

Fu,Y., Galan,J.E. (1999) A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion *Nature* **401**: 293-297.

Gal-Mor,O., Segal,G. (2003) Identification of CpxR as a positive regulator of icm and dot virulence genes of Legionella pneumophila *J.Bacteriol.* **185**: 4908-4919.

Galan,J.E. (1996a) Molecular and cellular bases of Salmonella entry into host cells *Curr.Top.Microbiol.Immunol.* **209:43-60.**: 43-60.

Galan,J.E. (1996b) Molecular genetic bases of Salmonella entry into host cells *Mol.Microbiol.* **20**: 263-271.

Galan,J.E., Curtiss,R., III (1989) Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells *Proc.Natl.Acad.Sci.U.S.A* **86**: 6383-6387.

Galan,J.E., Fu,Y. (2000) Modulation of actin cytoskeleton by Salmonella GTPase activating protein SptP *Methods Enzymol.* **325:496-504.**: 496-504.

Galperin,M.Y., Nikolskaya,A.N., and Koonin,E.V. (2001) Novel domains of the prokaryotic two-component signal transduction systems *FEMS Microbiol.Lett.* **203**: 11-21.

Garcia,V.E., Soncini,F.C., and Groisman,E.A. (1994) The role of the PhoP/PhoQ regulon in Salmonella virulence *Res.Microbiol.* **145**: 473-480.

Garcia-del Portillo,F., Finlay,B.B. (1994) Invasion and intracellular proliferation of Salmonella within non-phagocytic cells *Microbiologia* **10**: 229-238.

Garcia-del Portillo,F., Foster,J.W., and Finlay,B.B. (1993) Role of acid tolerance response genes in Salmonella typhimurium virulence *Infect.Immun.* **61**: 4489-4492.

Garcia-del Portillo,F., Zwick,M.B., Leung,K.Y., and Finlay,B.B. (1993) Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells *Proc.Natl.Acad.Sci.U.S.A* **90**: 10544-10548.

Garmendia,J., Beuzon,C.R., Ruiz-Albert,J., and Holden,D.W. (2003) The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the Salmonella typhimurium SPI-2 type III secretion system *Microbiology* **149**: 2385-2396.

Gaspar,J.A., Thomas,J.A., Marolda,C.L., and Valvano,M.A. (2000) Surface expression of O-specific lipopolysaccharide in Escherichia coli requires the function of the TolA protein *Mol.Microbiol.* **38**: 262-275.

Gebert,A., Jepson,M.A. (1996) Is the epithelial origin of M cells controversial? *Gastroenterology* **111**: 1163.

Germanier, R. and Fuer E. Isolation and charcterisation of GalE mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral vaccine. J Infect Dis 131, 553-558. 1975.
Ref Type: Generic

Geyer,R., Galanos,C., Westphal,O., and Golecki,J.R. (1979) A lipopolysaccharide-binding cell-surface protein from Salmonella minnesota. Isolation, partial characterization and occurrence in different Enterobacteriaceae *Eur.J.Biochem.* **98**: 27-38.

Gilman,R.H., Hornick,R.B., Woodard,W.E., DuPont,H.L., Snyder,M.J., Levine,M.M., and Libonati,J.P. (1977) Evaluation of a UDP-glucose-4-epimeraseless mutant of Salmonella typhi as a liver oral vaccine *J.Infect.Dis.* **136**: 717-723.

Ginocchio,C., Pace,J., and Galan,J.E. (1992) Identification and molecular characterization of a Salmonella typhimurium gene involved in triggering the internalization of salmonellae into cultured epithelial cells *Proc.Natl.Acad.Sci.U.S.A* **89**: 5976-5980.

Glazebrook,J., Ichige,A., and Walker,G.C. (1993) A Rhizobium meliloti homolog of the Escherichia coli peptide-antibiotic transport protein SbmA is essential for bacteroid development *Genes Dev.* **7**: 1485-1497.

Glazebrook,J., Ichige,A., and Walker,G.C. (1996) Genetic analysis of Rhizobium meliloti bacA-phoA fusion results in identification of degP: two loci required for symbiosis are closely linked to degP *J.Bacteriol.* **178**: 745-752.

Gleason,F.K., Holmgren,A. (1988) Thioredoxin and related proteins in procaryotes *FEMS Microbiol.Rev.* **4**: 271-297.

Goldberg,M.B., Rubin,R.H. (1988) The spectrum of Salmonella infection *Infect.Dis.Clin.North Am.* **2**: 571-598.

Gonzalez,C., Hone,D., Noriega,F.R., Tacket,C.O., Davis,J.R., Losonsky,G., Nataro,J.P., Hoffman,S., Malik,A., Nardin,E., and . (1994) Salmonella typhi vaccine strain CVD 908 expressing the circumsporozoite protein of Plasmodium falciparum: strain construction and safety and immunogenicity in humans *J.Infect.Dis.* **169**: 927-931.

Gottesman,S. (1996) Proteases and their targets in Escherichia coli *Annu.Rev.Genet.* **30:465-506.**: 465-506.

Gottesman,S., Roche,E., Zhou,Y., and Sauer,R.T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system *Genes Dev.* **12**: 1338-1347.

Govoni,G., Gros,P. (1998) Macrophage NRAMP1 and its role in resistance to microbial infections *Inflamm.Res.* **47**: 277-284.

Gray,C.W., Ward,R.V., Karran,E., Turconi,S., Rowles,A., Viglienghi,D., Southan,C., Barton,A., Fantom,K.G., West,A., Savopoulos,J., Hassan,N.J., Clinkenbeard,H., Hanning,C., Amegadzie,B., Davis,J.B., Dingwall,C., Livi,G.P., and Creasy,C.L. (2000) Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response *Eur.J.Biochem.* **267**: 5699-5710.

Griffith,K.L., Wolf,R.E., Jr. (2002) Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays *Biochem.Biophys.Res.Commun.* **290**: 397-402.

Groisman,E.A., Ochman,H. (1993) Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri *EMBO J.* **12**: 3779-3787.

Gross,C.A., Chan,C.L., and Lonetto,M.A. (1996) A structure/function analysis of Escherichia coli RNA polymerase *Philos.Trans.R.Soc.Lond B Biol.Sci.* **351**: 475-482.

Grossman,A.D., Erickson,J.W., and Gross,C.A. (1984) The htpR gene product of E. coli is a sigma factor for heat-shock promoters *Cell* **38**: 383-390.

Gubbins,M.J., Lau,I., Will,W.R., Manchak,J.M., Raivio,T.L., and Frost,L.S. (2002) The positive regulator, TraJ, of the Escherichia coli F plasmid is unstable in a cpxA* background *J.Bacteriol.* **184**: 5781-5788.

Guiney,D.G., Libby,S., Fang,F.C., Krause,M., and Fierer,J. (1995) Growth-phase regulation of plasmid virulence genes in Salmonella *Trends Microbiol.* **3**: 275-279.

Gulig,P.A. (1990) Virulence plasmids of Salmonella typhimurium and other salmonellae *Microb.Pathog.* **8**: 3-11.

Gulig,P.A., Danbara,H., Guiney,D.G., Lax,A.J., Norel,F., and Rhen,M. (1993) Molecular analysis of spv virulence genes of the Salmonella virulence plasmids *Mol.Microbiol.* **7**: 825-830.

Gupta,S.D., Lee,B.T., Camakaris,J., and Wu,H.C. (1995) Identification of cutC and cutF (nlpE) genes involved in copper tolerance in Escherichia coli *J.Bacteriol.* **177**: 4207-4215.

Hall,H.K., Foster,J.W. (1996) The role of fur in the acid tolerance response of Salmonella typhimurium is physiologically and genetically separable from its role in iron acquisition *J.Bacteriol.* **178**: 5683-5691.

Halsey,T.A., Vazquez-Torres,A., Gravdahl,D.J., Fang,F.C., and Libby,S.J. (2004) The ferritin-like Dps protein is required for Salmonella enterica serovar Typhimurium oxidative stress resistance and virulence *Infect.Immun.* **72**: 1155-1158.

Hardt,W.D., Galan,J.E. (1997) A secreted Salmonella protein with homology to an avirulence determinant of plant pathogenic bacteria *Proc.Natl.Acad.Sci.U.S.A* **94**: 9887-9892.

Hardt,W.D., Urlaub,H., and Galan,J.E. (1998) A substrate of the centisome 63 type III protein secretion system of Salmonella typhimurium is encoded by a cryptic bacteriophage *Proc.Natl.Acad.Sci.U.S.A* **95**: 2574-2579.

Harrison,J.A., Villarreal-Ramos,B., Mastroeni,P., Demarco,d.H., and Hormaeche,C.E. (1997) Correlates of protection induced by live Aro- Salmonella typhimurium vaccines in the murine typhoid model *Immunology* **90**: 618-625.

Hayashi,F., Smith,K.D., Ozinsky,A., Hawn,T.R., Yi,E.C., Goodlett,D.R., Eng,J.K., Akira,S., Underhill,D.M., and Aderem,A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5 *Nature* **410**: 1099-1103.

Hayward,R.D., Koronakis,V. (1999) Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella *EMBO J.* **18**: 4926-4934.

Hein,W.R. (1999) Organization of mucosal lymphoid tissue *Curr.Top.Microbiol.Immunol.* **236**: 1-15.

Heiskanen,P., Taira,S., and Rhen,M. (1994) Role of rpoS in the regulation of Salmonella plasmid virulence (spv) genes *FEMS Microbiol.Lett.* **123**: 125-130.

Helmann,J.D., Chamberlin,M.J. (1988) Structure and function of bacterial sigma factors *Annu.Rev.Biochem.* **57:839-72.**: 839-872.

Hengge-Aronis,R. (1993) Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in E. coli *Cell* **72**: 165-168.

Hengge-Aronis,R. (2002a) Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase *Microbiol.Mol.Biol.Rev.* **66**: 373-95, table.

Hengge-Aronis,R. (2002b) Stationary phase gene regulation: what makes an Escherichia coli promoter sigmaS-selective? *Curr.Opin.Microbiol.* **5**: 591-595.

Hensel,M., Shea,J.E., Gleeson,C., Jones,M.D., Dalton,E., and Holden,D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection *Science* **269**: 400-403.

Hensel,M., Shea,J.E., Raupach,B., Monack,D., Falkow,S., Gleeson,C., Kubo,T., and Holden,D.W. (1997) Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2 *Mol.Microbiol.* **24**: 155-167.

Hensel,M., Shea,J.E., Waterman,S.R., Mundy,R., Nikolaus,T., Banks,G., Vazquez-Torres,A., Gleeson,C., Fang,F.C., and Holden,D.W. (1998) Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages *Mol.Microbiol.* **30**: 163-174.

Hermant,D., Menard,R., Arricau,N., Parsot,C., and Popoff,M.Y. (1995) Functional conservation of the Salmonella and Shigella effectors of entry into epithelial cells *Mol.Microbiol.* **17**: 781-789.

Hersh,D., Monack,D.M., Smith,M.R., Ghori,N., Falkow,S., and Zychlinsky,A. (1999) The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1 *Proc.Natl.Acad.Sci.U.S.A* **96**: 2396-2401.

Heungens,K., Cowles,C.E., and Goodrich-Blair,H. (2002) Identification of Xenorhabdus nematophila genes required for mutualistic colonization of Steinernema carpocapsae nematodes *Mol.Microbiol.* **45**: 1337-1353.

Heusipp,G., Schmidt,M.A., and Miller,V.L. (2003) Identification of rpoE and nadB as host responsive elements of Yersinia enterocolitica *FEMS Microbiol.Lett.* **226**: 291-298.

Hild,E., Takayama,K., Olsson,R.M., and Kjelleberg,S. (2000) Evidence for a role of rpoE in stressed and unstressed cells of marine Vibrio angustum strain S14 *J.Bacteriol.* **182**: 6964-6974.

Hiles,I.D., Gallagher,M.P., Jamieson,D.J., and Higgins,C.F. (1987) Molecular characterization of the oligopeptide permease of Salmonella typhimurium *J.Mol.Biol.* **195**: 125-142.

Hindle,Z., Chatfield,S.N., Phillimore,J., Bentley,M., Johnson,J., Cosgrove,C.A., Ghaem-Maghami,M., Sexton,A., Khan,M., Brennan,F.R., Everest,P., Wu,T., Pickard,D., Holden,D.W., Dougan,G., Griffin,G.E., House,D., Santangelo,J.D., Khan,S.A., Shea,J.E., Feldman,R.G., and Lewis,D.J. (2002) Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers *Infect.Immun.* **70**: 3457-3467.

Hinton,J.C. (1997) The Escherichia coli genome sequence: the end of an era or the start of the FUN? *Mol.Microbiol.* **26**: 417-422.

Hiratsu,K., Amemura,M., Nashimoto,H., Shinagawa,H., and Makino,K. (1995) The rpoE gene of Escherichia coli, which encodes sigma E, is essential for bacterial growth at high temperature *J Bacteriol.* **177**: 2918-2922.

Hohmann,A.W., Schmidt,G., and Rowley,D. (1978) Intestinal colonization and virulence of Salmonella in mice *Infect.Immun.* **22**: 763-770.

Hoiseth,S.K., Stocker,B.A. (1981) Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines *Nature* **291**: 238-239.

Holck,A., Kleppe,K. (1988) Cloning and sequencing of the gene for the DNA-binding 17K protein of Escherichia coli *Gene* **67**: 117-124.

Holmgren,A. (1985) Thioredoxin *Annu.Rev.Biochem.* **54:237-71.**: 237-271.

Holmgren,A. (1989) Thioredoxin and glutaredoxin systems *J.Biol.Chem.* **264**: 13963-13966.

Hone,D.M., Attridge,S.R., Forrest,B., Morona,R., Daniels,D., LaBrooy,J.T., Bartholomeusz,R.C., Shearman,D.J., and Hackett,J. (1988) A galE via (Vi antigen-negative) mutant of Salmonella typhi Ty2 retains virulence in humans *Infect.Immun.* **56**: 1326-1333.

Hone,D.M., Harris,A.M., Chatfield,S., Dougan,G., and Levine,M.M. (1991) Construction of genetically defined double aro mutants of Salmonella typhi *Vaccine* **9**: 810-816.

Hoops,P., Prather,N.E., Berry,J., and Ravel,J.M. (1976) Evidence for an extrinsic immunogen in effective ribosomal vaccines from Salmonella typhimurium *Infect.Immun.* **13**: 1184-1192.

Horne,S.M., Kottom,T.J., Nolan,L.K., and Young,K.D. (1997) Decreased intracellular survival of an fkpA mutant of Salmonella typhimurium Copenhagen *Infect Immun.* **65**: 806-810.

Hornick,R.B. (1970) Pathogenesis of typhoid fever *J.Egypt.Public Health Assoc.* **45**: 247-259.

Hornick,R.B., Greisman,S.E., Woodward,T.E., DuPont,H.L., Dawkins,A.T., and Snyder,M.J. (1970a) Typhoid fever: pathogenesis and immunologic control *N.Engl.J.Med.* **283**: 686-691.

Hornick,R.B., Greisman,S.E., Woodward,T.E., DuPont,H.L., Dawkins,A.T., and Snyder,M.J. (1970b) Typhoid fever: pathogenesis and immunologic control. 2 *N.Engl.J.Med.* **283**: 739-746.

Hueck,C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants *Microbiol.Mol.Biol.Rev.* **62**: 379-433.

Hughes,K.T., Mathee,K. (1998) The anti-sigma factors *Annu.Rev.Microbiol.* **52**: 231-286.

Humphreys,S., Rowley,G., Stevenson,A., Anjum,M.F., Woodward,M.J., Gilbert,S., Kormanec,J., and Roberts,M. (2004) Role of the two-component regulator CpxAR in the virulence of Salmonella enterica serotype Typhimurium *Infect.Immun.* **72**: 4654-4661.

Humphreys,S., Rowley,G., Stevenson,A., Kenyon,W.J., Spector,M.P., and Roberts,M. (2003) Role of periplasmic peptidylprolyl isomerases in Salmonella enterica serovar Typhimurium virulence *Infect.Immun.* **71**: 5386-5388.

Humphreys,S., Stevenson,A., Bacon,A., Weinhardt,A.B., and Roberts,M. (1999) The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium Infect Immun* **67**: 1560-1568.

Ichige,A., Walker,G.C. (1997) Genetic analysis of the Rhizobium meliloti bacA gene: functional interchangeability with the Escherichia coli sbmA gene and phenotypes of mutants *J.Bacteriol.* **179**: 209-216.

Ishihama,A. (2000) Functional modulation of Escherichia coli RNA polymerase *Annu.Rev.Microbiol.* **54:499-518.**: 499-518.

Janeway,C.A., Jr., Medzhitov,R. (2002) Innate immune recognition *Annu.Rev.Immunol.* **20:197-216. Epub;%2001 Oct 04.**: 197-216.

Jepson,M.A., Clark,M.A. (1998) Studying M cells and their role in infection *Trends Microbiol.* **6**: 359-365.

Jepson,M.A., Clark,M.A. (2001) The role of M cells in Salmonella infection *Microbes.Infect* **3**: 1183-1190.

Jishage,M., Ishihama,A. (1995) Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of sigma 70 and sigma 38 *J.Bacteriol.* **177**: 6832-6835.

Johnson,K., Charles,I., Dougan,G., Pickard,D., O'Gaora,P., Costa,G., Ali,T., Miller,I., and Hormaeche,C. (1991) The role of a stress-response protein in Salmonella typhimurium virulence *Mol.Microbiol* **5**: 401-407.

Johnston,C., Pegues,D.A., Hueck,C.J., Lee,A., and Miller,S.I. (1996) Transcriptional activation of Salmonella typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily *Mol.Microbiol.* **22**: 715-727.

Jones,B.D., Falkow,S. (1996) Salmonellosis: host immune responses and bacterial virulence determinants *Annu.Rev.Immunol.* **14**: 533-561.

Jones,B.D., Ghori,N., and Falkow,S. (1994) Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches *J.Exp.Med.* **180**: 15-23.

Jones,J.M., Welty,D.J., and Nakai,H. (1998) Versatile action of Escherichia coli ClpXP as protease or molecular chaperone for bacteriophage Mu transposition *J.Biol.Chem.* **273**: 459-465.

Journet,L., Rigal,A., Lazdunski,C., and Benedetti,H. (1999) Role of TolR N-terminal, central, and C-terminal domains in dimerization and interaction with TolA and tolQ *J.Bacteriol.* **181**: 4476-4484.

Jovanovic,G., Weiner,L., and Model,P. (1996) Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the Escherichia coli stress-induced psp operon *J.Bacteriol.* **178**: 1936-1945.

Kallipolitis,B.H., Valentin-Hansen,P. (1998) Transcription of rpoH, encoding the Escherichia coli heat-shock regulator sigma32, is negatively controlled by the cAMP-CRP/CytR nucleoprotein complex *Mol.Microbiol.* **29**: 1091-1099.

Kanehara,K., Akiyama,Y., and Ito,K. (2001) Characterization of the yaeL gene product and its S2P-protease motifs in Escherichia coli *Gene* **281**: 71-79.

Kanehara,K., Ito,K., and Akiyama,Y. (2002) YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA *Genes Dev.* **16**: 2147-2155.

Kanehara,K., Ito,K., and Akiyama,Y. (2003) YaeL proteolysis of RseA is controlled by the PDZ domain of YaeL and a Gln-rich region of RseA *EMBO J.* **22**: 6389-6398.

Kaniga,K., Bossio,J.C., and Galan,J.E. (1994) The Salmonella typhimurium invasion genes invF and invG encode homologues of the AraC and PulD family of proteins *Mol.Microbiol.* **13**: 555-568.

Kaniga,K., Trollinger,D., and Galan,J.E. (1995) Identification of two targets of the type III protein secretion system encoded by the inv and spa loci of Salmonella typhimurium that have homology to the Shigella IpaD and IpaA proteins *J.Bacteriol.* **177**: 7078-7085.

Kaniga,K., Tucker,S., Trollinger,D., and Galan,J.E. (1995) Homologs of the Shigella IpaB and IpaC invasins are required for Salmonella typhimurium entry into cultured epithelial cells *J.Bacteriol.* **177**: 3965-3971.

Kaniga,K., Uralil,J., Bliska,J.B., and Galan,J.E. (1996) A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen Salmonella typhimurium *Mol.Microbiol.* **21**: 633-641.

Kannenberg,E.L., Carlson,R.W. (2001) Lipid A and O-chain modifications cause Rhizobium lipopolysaccharides to become hydrophobic during bacteroid development *Mol.Microbiol.* **39**: 379-391.

Kantele,A., Arvilommi,H., and Jokinen,I. (1986) Specific immunoglobulin-secreting human blood cells after peroral vaccination against Salmonella typhi *J.Infect.Dis.* **153**: 1126-1131.

Kato,J., Chu,L., Kitano,K., DeVault,J.D., Kimbara,K., Chakrabarty,A.M., and Misra,T.K. (1989) Nucleotide sequence of a regulatory region controlling alginate synthesis in Pseudomonas aeruginosa: characterization of the algR2 gene *Gene* **84**: 31-38.

KAUFFMANN,F. (1957) [The Kauffmann-White schema; diagnostic Salmonella antigen schema.] *Ergeb.Mikrobiol.Immunitatsforsch.Exp.Ther.* **30**: 160-216.

KAUFFMANN,F., EDWARDS,P.R. (1957) A revised, simplified Kauffmann-White schema *Acta Pathol.Microbiol.Scand.* **41**: 242-246.

Kelly,A., Goldberg,M.D., Carroll,R.K., Danino,V., Hinton,J.C., and Dorman,C.J. (2004) A global role for Fis in the transcriptional control of metabolism and type III secretion in Salmonella enterica serovar Typhimurium *Microbiology* **150**: 2037-2053.

Kenyon,W.J., Sayers,D.G., Humphreys,S., Roberts,M., and Spector,M.P. (2002) The starvation-stress response of Salmonella enterica serovar Typhimurium requires sigma(E)-, but not CpxR-regulated extracytoplasmic functions *Microbiology* **148**: 113-122.

Kim,S.K., Wilmes-Riesenberg,M.R., and Wanner,B.L. (1996) Involvement of the sensor kinase EnvZ in the in vivo activation of the response-regulator PhoB by acetyl phosphate *Mol.Microbiol.* **22**: 135-147.

Kim,S.Y., Nam,T.W., Shin,D., Koo,B.M., Seok,Y.J., and Ryu,S. (1999) Purification of Mlc and analysis of its effects on the pts expression in Escherichia coli *J.Biol.Chem.* **274**: 25398-25402.

Kimbara,K., Chakrabarty,A.M. (1989) Control of alginate synthesis in Pseudomonas aeruginosa: regulation of the algR1 gene *Biochem.Biophys.Res.Commun.* **164**: 601-608.

Kimbrough,T.G., Miller,S.I. (2000) Contribution of Salmonella typhimurium type III secretion components to needle complex formation *Proc.Natl.Acad.Sci.U.S.A* **97**: 11008-11013.

Kitagawa,M., Miyakawa,M., Matsumura,Y., and Tsuchido,T. (2002) Escherichia coli small heat shock proteins, IbpA and IbpB, protect enzymes from inactivation by heat and oxidants *Eur.J.Biochem.* **269**: 2907-2917.

Klugman,K.P., Koornhof,H.J., Robbins,J.B., and Le Cam,N.N. (1996) Immunogenicity, efficacy and serological correlate of protection of Salmonella typhi Vi capsular polysaccharide vaccine three years after immunization *Vaccine* **14**: 435-438.

Knodler,L.A., Celli,J., Hardt,W.D., Vallance,B.A., Yip,C., and Finlay,B.B. (2002) Salmonella effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems *Mol.Microbiol.* **43**: 1089-1103.

Knodler,L.A., Vallance,B.A., Hensel,M., Jackel,D., Finlay,B.B., and Steele-Mortimer,O. (2003) Salmonella type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes *Mol.Microbiol.* **49**: 685-704.

Knuth,K., Niesalla,H., Hueck,C.J., and Fuchs,T.M. (2004) Large-scale identification of essential Salmonella genes by trapping lethal insertions *Mol.Microbiol.* **51**: 1729-1744.

Koch,C., Hoiby,N. (1993) Pathogenesis of cystic fibrosis *Lancet* **341**: 1065-1069.

Kohbata,S., Yokoyama,H., and Yabuuchi,E. (1986) Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study *Microbiol.Immunol.* **30**: 1225-1237.

Konadu,E.Y., Lin,F.Y., Ho,V.A., Thuy,N.T., Van Bay,P., Thanh,T.C., Khiem,H.B., Trach,D.D., Karpas,A.B., Li,J., Bryla,D.A., Robbins,J.B., and Szu,S.C. (2000) Phase 1 and phase 2 studies of Salmonella enterica serovar paratyphi A O-specific polysaccharide-tetanus toxoid conjugates in adults, teenagers, and 2- to 4-year-old children in Vietnam *Infect Immun.* **68**: 1529-1534.

Kormanec,J. (2001) Analyzing the developmental expression of sigma factors with S1-nuclease mapping *Methods Mol.Biol.* **160**: 481-494.

Korndorfer,I.P., Dommel,M.K., and Skerra,A. (2004) Structure of the periplasmic chaperone Skp suggests functional similarity with cytosolic chaperones despite differing architecture *Nat.Struct.Mol.Biol.*

Koski,P., Hirvas,L., and Vaara,M. (1990) Complete sequence of the ompH gene encoding the 16-kDa cationic outer membrane protein of Salmonella typhimurium *Gene* **88**: 117-120.

Koski,P., Rhen,M., Kantele,J., and Vaara,M. (1989) Isolation, cloning, and primary structure of a cationic 16-kDa outer membrane protein of Salmonella typhimurium *J.Biol.Chem.* **264**: 18973-18980.

Kossaczka,Z., Lin,F.Y., Ho,V.A., Thuy,N.T., Van Bay,P., Thanh,T.C., Khiem,H.B., Trach,D.D., Karpas,A., Hunt,S., Bryla,D.A., Schneerson,R., Robbins,J.B., and Szu,S.C. (1999) Safety and immunogenicity of Vi conjugate vaccines for typhoid fever in adults, teenagers, and 2- to 4-year-old children in Vietnam *Infect Immun.* **67**: 5806-5810.

Kovacikova,G., Skorupski,K. (2002) The alternative sigma factor sigma(E) plays an important role in intestinal survival and virulence in Vibrio cholerae *Infect.Immun.* **70**: 5355-5362.

Kowarz,L., Coynault,C., Robbe-Saule,V., and Norel,F. (1994) The Salmonella typhimurium katF (rpoS) gene: cloning, nucleotide sequence, and regulation of spvR and spvABCD virulence plasmid genes *J.Bacteriol.* **176**: 6852-6860.

Kraehenbuhl,J.P., Neutra,M.R. (2000) Epithelial M cells: differentiation and function *Annu.Rev.Cell Dev.Biol.* **16:301-32.**: 301-332.

Krojer,T., Garrido-Franco,M., Huber,R., Ehrmann,M., and Clausen,T. (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine *Nature* **416**: 455-459.

Kubori,T., Matsushima,Y., Nakamura,D., Uralil,J., Lara-Tejero,M., Sukhan,A., Galan,J.E., and Aizawa,S.I. (1998) Supramolecular structure of the Salmonella typhimurium type III protein secretion system *Science* **280**: 602-605.

Kuczynska-Wisnik,D., Kedzierska,S., Matuszewska,E., Lund,P., Taylor,A., Lipinska,B., and Laskowska,E. (2002) The Escherichia coli small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock *Microbiology* **148**: 1757-1765.

Kuusi,N., Nurminen,M., Saxen,H., Valtonen,M., and Makela,P.H. (1979) Immunization with major outer membrane proteins in experimental salmonellosis of mice *Infect.Immun.* **25**: 857-862.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature* **227**: 680-685.

Lange,R., Barth,M., and Hengge-Aronis,R. (1993) Complex transcriptional control of the sigma s-dependent stationary-phase-induced and osmotically regulated osmY (csi-5) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary-phase response of Escherichia coli *J.Bacteriol.* **175**: 7910-7917.

Lange,R., Fischer,D., and Hengge-Aronis,R. (1995) Identification of transcriptional start sites and the role of ppGpp in the expression of rpoS, the structural gene for the sigma S subunit of RNA polymerase in Escherichia coli *J.Bacteriol.* **177**: 4676-4680.

Lange,R., Hengge-Aronis,R. (1991) Identification of a central regulator of stationary-phase gene expression in Escherichia coli *Mol.Microbiol.* **5**: 49-59.

Lathe,R., Buc,H., Lecocq,J.P., and Bautz,E.K. (1980) Prokaryotic histone-like protein interacting with RNA polymerase *Proc.Natl.Acad.Sci.U.S.A* **77**: 3548-3552.

Lavina,M., Pugsley,A.P., and Moreno,F. (1986) Identification, mapping, cloning and characterization of a gene (sbmA) required for microcin B17 action on Escherichia coli K12 *J.Gen.Microbiol.* **132 (Pt 6)**: 1685-1693.

Lazar,S.W., Kolter,R. (1996) SurA assists the folding of Escherichia coli outer membrane proteins *J.Bacteriol.* **178**: 1770-1773.

Lazzaroni,J.C., Dubuisson,J.F., and Vianney,A. (2002) The Tol proteins of Escherichia coli and their involvement in the translocation of group A colicins *Biochimie* **84**: 391-397.

Lazzaroni,J.C., Fognini-Lefebvre,N., and Portalier,R. (1989) Cloning of the excC and excD genes involved in the release of periplasmic proteins by Escherichia coli K12 *Mol.Gen.Genet.* **218**: 460-464.

Le Minor,L., Bockemuhl,J. (1988) [1987 supplement (no. 31) to the schema of Kauffmann-White] *Ann.Inst.Pasteur Microbiol.* **139**: 331-335.

Le Minor,L., Veron,M., and Popoff,M. (1982) [A proposal for Salmonella nomenclature] *Ann.Microbiol.(Paris)* **133**: 245-254.

Leclerc,G.J., Tartera,C., and Metcalf,E.S. (1998) Environmental regulation of Salmonella typhi invasion-defective mutants *Infect Immun.* **66**: 682-691.

Lee,A.H., Zareei,M.P., and Daefler,S. (2002) Identification of a NIPSNAP homologue as host cell target for Salmonella virulence protein SpiC *Cell Microbiol.* **4**: 739-750.

Lee,C.A., Falkow,S. (1990) The ability of Salmonella to enter mammalian cells is affected by bacterial growth state *Proc.Natl.Acad.Sci.U.S.A* **87**: 4304-4308.

Lee,C.A., Jones,B.D., and Falkow,S. (1992) Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants *Proc.Natl.Acad.Sci.U.S.A* **89**: 1847-1851.

Lencer,W.I., Neutra,M.R. (2000) Salmonella pathogenesis: the trojan horse or the New York shuttle? *Gastroenterology* **118**: 803-805.

Levengood,S.K., Beyer,W.F., Jr., and Webster,R.E. (1991) TolA: a membrane protein involved in colicin uptake contains an extended helical region *Proc.Natl.Acad.Sci.U.S.A* **88**: 5939-5943.

LeVier,K., Phillips,R.W., Grippe,V.K., Roop,R.M., and Walker,G.C. (2000) Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival *Science* **287**: 2492-2493.

Levine,M.M., Ferreccio,C., Black,R.E., and Germanier,R. (1987) Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation *Lancet* **1**: 1049-1052.

Levine,M.M., Galen,J., Barry,E., Noriega,F., Chatfield,S., Sztein,M., Dougan,G., and Tacket,C. (1996) Attenuated Salmonella as live oral vaccines against typhoid fever and as live vectors *J Biotechnol.* **44**: 193-196.

Li,K., Pasternak,C., Hartig,E., Haberzettl,K., Maxwell,A., and Klug,G. (2004) Thioredoxin can influence gene expression by affecting gyrase activity *Nucleic Acids Res.* **32**: 4563-4575.

Li,W., Srinivasula,S.M., Chai,J., Li,P., Wu,J.W., Zhang,Z., Alnemri,E.S., and Shi,Y. (2002) Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi *Nat.Struct.Biol.* **9**: 436-441.

Lin,S.L., Le,T.X., and Cowen,D.S. (2003) SptP, a Salmonella typhimurium type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation *Cell Microbiol.* **5**: 267-275.

Linn,T., St Pierre,R. (1990) Improved vector system for constructing transcriptional fusions that ensures independent translation of lacZ *J.Bacteriol.* **172**: 1077-1084.

Lipinska,B., Sharma,S., and Georgopoulos,C. (1988) Sequence analysis and regulation of the htrA gene of Escherichia coli: a sigma 32-independent mechanism of heat-inducible transcription *Nucleic.Acids.Res.* **16**: 10053-10067.

Lizewski,S.E., Lundberg,D.S., and Schurr,M.J. (2002) The transcriptional regulator AlgR is essential for Pseudomonas aeruginosa pathogenesis *Infect.Immun.* **70**: 6083-6093.

Lizewski,S.E., Schurr,J.R., Jackson,D.W., Frisk,A., Carterson,A.J., and Schurr,M.J. (2004) Identification of AlgR-regulated genes in Pseudomonas aeruginosa by use of microarray analysis *J.Bacteriol.* **186**: 5672-5684.

Lo,W.F., Ong,H., Metcalf,E.S., and Soloski,M.J. (1999) T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules *J.Immunol.* **162**: 5398-5406.

Loewen,P.C., Hengge-Aronis,R. (1994) The role of the sigma factor sigma S (KatF) in bacterial global regulation *Annu.Rev.Microbiol.* **48:53-80.**: 53-80.

Loewen,P.C., Hu,B., Strutinsky,J., and Sparling,R. (1998) Regulation in the rpoS regulon of Escherichia coli *Can.J.Microbiol.* **44**: 707-717.

Loewen,P.C., von,O., I, Switala,J., and Mulvey,M.R. (1993) KatF (sigma S) synthesis in Escherichia coli is subject to posttranscriptional regulation *J.Bacteriol.* **175**: 2150-2153.

Lonetto,M., Gribskov,M., and Gross,C.A. (1992) The sigma 70 family: sequence conservation and evolutionary relationships *J.Bacteriol.* **174**: 3843-3849.

Lonetto,M.A., Brown,K.L., Rudd,K.E., and Buttner,M.J. (1994) Analysis of the Streptomyces coelicolor sigE gene reveals the existence of a subfamily of eubacterial

RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions *Proc.Natl.Acad.Sci.U.S.A* **91**: 7573-7577.

Long,S., McCune,S., and Walker,G.C. (1988) Symbiotic loci of Rhizobium meliloti identified by random TnphoA mutagenesis *J.Bacteriol.* **170**: 4257-4265.

Lorenz,M.G., Wackernagel,W. (1994) Bacterial gene transfer by natural genetic transformation in the environment *Microbiol.Rev.* **58**: 563-602.

Lostroh,C.P., Lee,C.A. (2001) The Salmonella pathogenicity island-1 type III secretion system *Microbes.Infect.* **3**: 1281-1291.

Lucas,R.L., Lee,C.A. (2001) Roles of hilC and hilD in regulation of hilA expression in Salmonella enterica serovar Typhimurium *J.Bacteriol.* **183**: 2733-2745.

Madara,J.L., Nash,S., Moore,R., and Atisook,K. (1990) Structure and function of the intestinal epithelial barrier in health and disease *Monogr Pathol.*: 306-324.

Marcus,S.L., Brumell,J.H., Pfeifer,C.G., and Finlay,B.B. (2000) Salmonella pathogenicity islands: big virulence in small packages *Microbes.Infect.* **2**: 145-156.

Martin,D.W., Holloway,B.W., and Deretic,V. (1993) Characterization of a locus determining the mucoid status of Pseudomonas aeruginosa: AlgU shows sequence similarities with a Bacillus sigma factor *J.Bacteriol.* **175**: 1153-1164.

Martin,D.W., Schurr,M.J., Mudd,M.H., Govan,J.R., Holloway,B.W., and Deretic,V. (1993) Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients *Proc.Natl.Acad.Sci.U.S.A* **90**: 8377-8381.

Martin,D.W., Schurr,M.J., Yu,H., and Deretic,V. (1994) Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in Pseudomonas aeruginosa: relationship to sigma E and stress response *J Bacteriol.* **176**: 6688-6696.

Martinez-Moya,M., de Pedro,M.A., Schwarz,H., and Garcia-del Portillo,F. (1998) Inhibition of Salmonella intracellular proliferation by non-phagocytic eucaryotic cells *Res.Microbiol.* **149**: 309-318.

Mastroeni,P., Menager,N. (2003) Development of acquired immunity to Salmonella *J.Med.Microbiol.* **52**: 453-459.

Mathee,K., McPherson,C.J., and Ohman,D.E. (1997) Posttranslational control of the algT (algU)-encoded sigma22 for expression of the alginate regulon in Pseudomonas aeruginosa and localization of its antagonist proteins MucA and MucB (AlgN) *J.Bacteriol.* **179**: 3711-3720.

Matsubara,M., Kitaoka,S.I., Takeda,S.I., and Mizuno,T. (2000) Tuning of the porin expression under anaerobic growth conditions by his-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in Escherichia coli *Genes Cells* **5**: 555-569.

Matsubara,M., Mizuno,T. (1999) EnvZ-independent phosphotransfer signaling pathway of the OmpR-mediated osmoregulatory expression of OmpC and OmpF in Escherichia coli *Biosci.Biotechnol.Biochem.* **63**: 408-414.

Maury,J., Nicoletti,C., Guzzo-Chambraud,L., and Maroux,S. (1995) The filamentous brush border glycocalyx, a mucin-like marker of enterocyte hyper-polarization *Eur.J.Biochem.* **228**: 323-331.

McCann,M.P., Fraley,C.D., and Matin,A. (1993) The putative sigma factor KatF is regulated posttranscriptionally during carbon starvation *J.Bacteriol.* **175**: 2143-2149.

McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W., Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M., Du,F., Hou,S., Layman,D., Leonard,S., Nguyen,C., Scott,K., Holmes,A., Grewal,N., Mulvaney,E., Ryan,E., Sun,H., Florea,L., Miller,W., Stoneking,T., Nhan,M., Waterston,R., and Wilson,R.K. (2001) Complete genome sequence of Salmonella enterica serovar Typhimurium LT2 *Nature* **413**: 852-856.

McClelland,M., Wilson,R.K. (1998) Comparison of sample sequences of the Salmonella typhi genome to the sequence of the complete Escherichia coli K-12 genome *Infect.Immun.* **66**: 4305-4312.

McEwen,J., Silverman,P. (1980) Genetic analysis of Escherichia coli K-12 chromosomal mutants defective in expression of F-plasmid functions: identification of genes cpxA and cpxB *J Bacteriol.* **144**: 60-67.

McEwen,J., Silverman,P.M. (1982) Mutations in genes cpxA and cpxB alter the protein composition of Escherichia coli inner and outer membranes *J Bacteriol.* **151**: 1553-1559.

McGhie,E.J., Hayward,R.D., and Koronakis,V. (2001) Cooperation between actin-binding proteins of invasive Salmonella: SipA potentiates SipC nucleation and bundling of actin *EMBO J.* **20**: 2131-2139.

Menard,R., Sansonetti,P.J., and Parsot,C. (1993) Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells *J.Bacteriol.* **175**: 5899-5906.

Merrick,M., Gibbins,J., and Toukdarian,A. (1987) The nucleotide sequence of the sigma factor gene ntrA (rpoN) of Azotobacter vinelandii: analysis of conserved sequences in NtrA proteins *Mol.Gen.Genet.* **210**: 323-330.

Merrick,M.J. (1993) In a class of its own--the RNA polymerase sigma factor sigma 54 (sigma N) *Mol.Microbiol.* **10**: 903-909.

Miao,E.A., Freeman,J.A., and Miller,S.I. (2002) Transcription of the SsrAB regulon is repressed by alkaline pH and is independent of PhoPQ and magnesium concentration *J.Bacteriol.* **184**: 1493-1497.

Miao,E.A., Miller,S.I. (2000) A conserved amino acid sequence directing intracellular type III secretion by Salmonella typhimurium *Proc.Natl.Acad.Sci.U.S.A* **%20;97**: 7539-7544.

Miller,J.H. (1972) *Experiments in Molecular Genetics* NY: Cold Spring Harbor Laboratory Press.

Miller,S.I. (1991) PhoP/PhoQ: macrophage-specific modulators of Salmonella virulence? *Mol.Microbiol* **5**: 2073-2078.

Mills,D.M., Bajaj,V., and Lee,C.A. (1995) A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome *Mol.Microbiol.* **15**: 749-759.

Minakhin,L., Nechaev,S., Campbell,E.A., and Severinov,K. (2001) Recombinant Thermus aquaticus RNA polymerase, a new tool for structure-based analysis of transcription *J.Bacteriol.* **183**: 71-76.

Missiakas,D., Betton,J.M., and Raina,S. (1996) New components of protein folding in extracytoplasmic compartments of Escherichia coli SurA, FkpA and Skp/OmpH *Mol.Microbiol* **21**: 871-884.

Missiakas,D., Mayer,M.P., Lemaire,M., Georgopoulos,C., and Raina,S. (1997) Modulation of the Escherichia coli sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins *Mol.Microbiol.* **24**: 355-371.

Missiakas,D., Raina,S. (1998) The extracytoplasmic function sigma factors: role and regulation *Mol.Microbiol* **28**: 1059-1066.

Miticka,H., Rezuchova,B., Homerova,D., Roberts,M., and Kormanec,J. (2004) Identification of nucleotides critical for activity of the sigma(E)-dependent rpoEp3 promoter in Salmonella enterica serovar Typhimurium *FEMS Microbiol.Lett.* **238**: 227-233.

Miticka,H., Rowley,G., Rezuchova,B., Homerova,D., Humphreys,S., Farn,J., Roberts,M., and Kormanec,J. (2003) Transcriptional analysis of the rpoE gene encoding extracytoplasmic stress response sigma factor sigmaE in Salmonella enterica serovar Typhimurium *FEMS Microbiol.Lett.* **226**: 307-314.

Model,P., Jovanovic,G., and Dworkin,J. (1997) The Escherichia coli phage-shock-protein (psp) operon *Mol.Microbiol.* **24**: 255-261.

Mohr,C.D., Leveau,J.H., Krieg,D.P., Hibler,N.S., and Deretic,V. (1992) AlgR-binding sites within the algD promoter make up a set of inverted repeats separated by a large intervening segment of DNA *J.Bacteriol.* **174**: 6624-6633.

Molbak,K., Baggesen,D.L., Aarestrup,F.M., Ebbesen,J.M., Engberg,J., Frydendahl,K., Gerner-Smidt,P., Petersen,A.M., and Wegener,H.C. (1999) An outbreak of multidrug-resistant, quinolone-resistant Salmonella enterica serotype typhimurium DT104 *N.Engl.J.Med.* **341**: 1420-1425.

Monack,D.M., Hersh,D., Ghori,N., Bouley,D., Zychlinsky,A., and Falkow,S. (2000) Salmonella exploits caspase-1 to colonize Peyer's patches in a murine typhoid model *J.Exp.Med.* **192**: 249-258.

Moreno,E., Stackebrandt,E., Dorsch,M., Wolters,J., Busch,M., and Mayer,H. (1990) Brucella abortus 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria *J.Bacteriol.* **172**: 3569-3576.

Morett,E., Buck,M. (1989) In vivo studies on the interaction of RNA polymerase-sigma 54 with the Klebsiella pneumoniae and Rhizobium meliloti nifH promoters. The role of NifA in the formation of an open promoter complex *J.Mol.Biol.* **210**: 65-77.

Morgan,E., Campbell,J.D., Rowe,S.C., Bispham,J., Stevens,M.P., Bowen,A.J., Barrow,P.A., Maskell,D.J., and Wallis,T.S. (2004) Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium *Mol.Microbiol.* **54**: 994-1010.

Muffler,A., Barth,M., Marschall,C., and Hengge-Aronis,R. (1997) Heat shock regulation of sigmaS turnover: a role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in Escherichia coli *J.Bacteriol.* **179**: 445-452.

Muller,M.M., Vianney,A., Lazzaroni,J.C., Webster,R.E., and Portalier,R. (1993) Membrane topology of the Escherichia coli TolR protein required for cell envelope integrity *J.Bacteriol.* **175**: 6059-6061.

Murdoch,D.A., Banatvaia,N., Bone,A., Shoismatulloev,B.I., Ward,L.R., and Threlfall,E.J. (1998) Epidemic ciprofloxacin-resistant Salmonella typhi in Tajikistan *Lancet* **351**: 339.

Murli,S., Watson,R.O., and Galan,J.E. (2001) Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of Salmonella with host cells *Cell Microbiol.* **3**: 795-810.

Nagai,H., Yano,R., Erickson,J.W., and Yura,T. (1990) Transcriptional regulation of the heat shock regulatory gene rpoH in Escherichia coli: involvement of a novel catabolite-sensitive promoter *J.Bacteriol.* **172**: 2710-2715.

Nagasawa,S., Ishige,K., and Mizuno,T. (1993) Novel members of the two-component signal transduction genes in Escherichia coli *J.Biochem.(Tokyo)* **114**: 350-357.

Nakamura,Y., Osawa,T., and Yura,T. (1977) Chromosomal location of a structural gene for the RNA polymerase sigma factor in Escherichia coli *Proc.Natl.Acad.Sci.U.S.A* **74**: 1831-1835.

Nakayama,S., Kushiro,A., Asahara,T., Tanaka,R., Hu,L., Kopecko,D.J., and Watanabe,H. (2003) Activation of hilA expression at low pH requires the signal sensor CpxA, but not the cognate response regulator CpxR, in Salmonella enterica serovar Typhimurium *Microbiology* **149**: 2809-2817.

Nakayama,S., Watanabe,H. (1998) Identification of cpxR as a positive regulator essential for expression of the Shigella sonnei virF gene *J Bacteriol.* **180**: 3522-3528.

Nardelli-Haefliger,D., Kraehenbuhl,J.P., Curtiss,R., III, Schodel,F., Potts,A., Kelly,S., and De Grandi,P. (1996) Oral and rectal immunization of adult female volunteers with a recombinant attenuated Salmonella typhi vaccine strain *Infect.Immun.* **64**: 5219-5224.

Nastasi,A., Mammina,C., and Salsa,L. (1999) Outbreak of Salmonella enteritis bongori 48:z35:- in Sicily *Euro.Surveill* **4**: 97-98.

Neidhardt, F. *Escherichia coli* and *Salmonella.* 2, 2691. 1996.
Ref Type: Generic

Neutra,M.R. (1999) M cells in antigen sampling in mucosal tissues *Curr.Top.Microbiol.Immunol.* **236**: 17-32.

Ninfa,A.J., Magasanik,B. (1986) Covalent modification of the glnG product, NRI, by the glnL product, NRII, regulates the transcription of the glnALG operon in Escherichia coli *Proc.Natl.Acad.Sci.U.S.A* **83**: 5909-5913.

Ninfa,A.J., Ninfa,E.G., Lupas,A.N., Stock,A., Magasanik,B., and Stock,J. (1988) Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of the Ntr regulon: evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism *Proc.Natl.Acad.Sci.U.S.A* **85**: 5492-5496.

Nitta,T., Nagamitsu,H., Murata,M., Izu,H., and Yamada,M. (2000) Function of the sigma(E) regulon in dead-cell lysis in stationary-phase Escherichia coli *J.Bacteriol.* **182**: 5231-5237.

Novakova,R., Sevcikova,B., and Kormanec,J. (1998) A method for the identification of promoters recognized by RNA polymerase containing a particular sigma factor: cloning of a developmentally regulated promoter and corresponding gene directed by the Streptomyces aureofaciens sigma factor RpoZ *Gene* **208**: 43-50.

O'Neal,C.R., Gabriel,W.M., Turk,A.K., Libby,S.J., Fang,F.C., and Spector,M.P. (1994) RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in Salmonella typhimurium *J.Bacteriol.* **176**: 4610-4616.

Ochman,H., Groisman,E.A. (1996) Distribution of pathogenicity islands in Salmonella spp *Infect.Immun.* **64**: 5410-5412.

Ochman,H., Soncini,F.C., Solomon,F., and Groisman,E.A. (1996) Identification of a pathogenicity island required for Salmonella survival in host cells *Proc.Natl.Acad.Sci.U.S.A* **93**: 7800-7804.

Ocholi,R.A., Enurah,L.U., and Odeyemi,P.S. (1987) Fatal case of salmonellosis (Salmonella pullorum) in a chimpanzee (Pan troglodytes) in the Jos Zoo *J.Wildl.Dis.* **23**: 669-670.

Orchard,S.S., Goodrich-Blair,H. (2004) Identification and functional characterization of a Xenorhabdus nematophila oligopeptide permease *Appl.Environ.Microbiol.* **70**: 5621-5627.

Oshima,T., Aiba,H., Masuda,Y., Kanaya,S., Sugiura,M., Wanner,B.L., Mori,H., and Mizuno,T. (2002) Transcriptome analysis of all two-component regulatory system mutants of Escherichia coli K-12 *Mol.Microbiol.* **46**: 281-291.

Otto,K., Silhavy,T.J. (2002) Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway *Proc.Natl.Acad.Sci.U.S.A* **99**: 2287-2292.

Ouellette,A.J. (1997) Paneth cells and innate immunity in the crypt microenvironment *Gastroenterology* **113**: 1779-1784.

Owen,R.L. (1994) M cells--entryways of opportunity for enteropathogens *J.Exp.Med.* **180**: 7-9.

Owen,R.L., Ermak,T.H. (1990) Structural specializations for antigen uptake and processing in the digestive tract *Springer Semin.Immunopathol.* **12**: 139-152.

Owen,R.L., Jones,A.L. (1974) Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles *Gastroenterology* **66**: 189-203.

Paget,M.S., Helmann,J.D. (2003) The sigma70 family of sigma factors *Genome Biol.* **4**: 203.

Pallen,M. (1999) RpoN-dependent transcription of rpoH? *Mol.Microbiol.* **31**: 393.

Pallen,M.J., Wren,B.W. (1997) The HtrA family of serine proteases *Mol.Microbiol* **26**: 209-221.

Pang,T., Bhutta,Z.A., Finlay,B.B., and Altwegg,M. (1995) Typhoid fever and other salmonellosis: a continuing challenge *Trends Microbiol.* **3**: 253-255.

Pang,T., Levine,M.M., Ivanoff,B., Wain,J., and Finlay,B.B. (1998) Typhoid fever--important issues still remain *Trends Microbiol.* **6**: 131-133.

Parkhill,J., Dougan,G., James,K.D., Thomson,N.R., Pickard,D., Wain,J., Churcher,C., Mungall,K.L., Bentley,S.D., Holden,M.T., Sebaihia,M., Baker,S., Basham,D., Brooks,K., Chillingworth,T., Connerton,P., Cronin,A., Davis,P., Davies,R.M., Dowd,L., White,N., Farrar,J., Feltwell,T., Hamlin,N., Haque,A., Hien,T.T., Holroyd,S., Jagels,K., Krogh,A., Larsen,T.S., Leather,S., Moule,S., O'Gaora,P., Parry,C., Quail,M., Rutherford,K., Simmonds,M., Skelton,J., Stevens,K., Whitehead,S., and Barrell,B.G. (2001) Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18 *Nature* **413**: 848-852.

Penheiter,K.L., Mathur,N., Giles,D., Fahlen,T., and Jones,B.D. (1997) Non-invasive Salmonella typhimurium mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches *Mol.Microbiol.* **24**: 697-709.

Pignato,S., Giammanco,G., Santangelo,C., and Giammanco,G.M. (1998) Endemic presence of Salmonella bongori 48:z35:- causing enteritis in children in Sicily *Res.Microbiol.* **149**: 429-431.

Pitel,F., Lantier,I., Riquet,J., Lanneluc,I., Tabet-Aoul,K., Saidi-Mehtar,N., Lantier,F., and Gellin,J. (1994) Cloning, sequencing, and localization of an ovine fragment of the NRAMP gene, a candidate for the ITY/LSH/BCG gene *Mamm.Genome* **5**: 834-835.

Plant,J., Glynn,A.A. (1974) Natural resistance to Salmonella infection, delayed hypersensitivity and Ir genes in different strains of mice *Nature* **248**: 345-347.

Plumbridge,J. (2001) DNA binding sites for the Mlc and NagC proteins: regulation of nagE, encoding the N-acetylglucosamine-specific transporter in Escherichia coli *Nucleic Acids Res.* **29**: 506-514.

Pogliano,J., Dong,J.M., De Wulf,P., Furlong,D., Boyd,D., Losick,R., Pogliano,K., and Lin,E.C. (1998) Aberrant cell division and random FtsZ ring positioning in Escherichia coli cpxA* mutants *J.Bacteriol.* **180**: 3486-3490.

Pogliano,J., Lynch,A.S., Belin,D., Lin,E.C., and Beckwith,J. (1997) Regulation of Escherichia coli cell envelope proteins involved in protein folding and degradation by the Cpx two-component system *Genes Dev.* **11**: 1169-1182.

Poltorak,A., He,X., Smirnova,I., Liu,M.Y., Van Huffel,C., Du,X., Birdwell,D., Alejos,E., Silva,M., Galanos,C., Freudenberg,M., Ricciardi-Castagnoli,P., Layton,B., and Beutler,B. (1998a) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene *Science* **282**: 2085-2088.

Poltorak,A., Smirnova,I., He,X., Liu,M.Y., Van Huffel,C., McNally,O., Birdwell,D., Alejos,E., Silva,M., Du,X., Thompson,P., Chan,E.K., Ledesma,J., Roe,B., Clifton,S., Vogel,S.N., and Beutler,B. (1998b) Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region *Blood Cells Mol.Dis.* **24**: 340-355.

Popham,D.L., Szeto,D., Keener,J., and Kustu,S. (1989) Function of a bacterial activator protein that binds to transcriptional enhancers *Science* **243**: 629-635.

Porwollik,S., Frye,J., Florea,L.D., Blackmer,F., and McClelland,M. (2003) A non-redundant microarray of genes for two related bacteria *Nucleic Acids Res.* **31**: 1869-1876.

Possot,O., d'Enfert,C., Reyss,I., and Pugsley,A.P. (1992) Pullulanase secretion in Escherichia coli K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein *Mol.Microbiol.* **6**: 95-105.

Prouty,A.M., Van Velkinburgh,J.C., and Gunn,J.S. (2002) Salmonella enterica serovar typhimurium resistance to bile: identification and characterization of the tolQRA cluster *J.Bacteriol.* **184**: 1270-1276.

Raetz,C.R. (1993) Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction *J.Bacteriol.* **175**: 5745-5753.

Raffa,R.G., Raivio,T.L. (2002) A third envelope stress signal transduction pathway in Escherichia coli *Mol.Microbiol.* **45**: 1599-1611.

Raffatellu,M., Wilson,R.P., Chessa,D., Andrews-Polymenis,H., Tran,Q.T., Lawhon,S., Khare,S., Adams,L.G., and Baumler,A.J. (2005) SipA, SopA, SopB, SopD, and SopE2 contribute to Salmonella enterica serotype typhimurium invasion of epithelial cells *Infect.Immun.* **73**: 146-154.

Rahfeld,J.U., Rucknagel,K.P., Schelbert,B., Ludwig,B., Hacker,J., Mann,K., and Fischer,G. (1994) Confirmation of the existence of a third family among peptidyl-prolyl cis/trans isomerases. Amino acid sequence and recombinant production of parvulin *FEBS Lett.* **352**: 180-184.

Raina,S., Missiakas,D., and Georgopoulos,C. (1995) The rpoE gene encoding the sigma E (sigma 24) heat shock sigma factor of Escherichia coli *EMBO J* **14**: 1043-1055.

Rainwater,S., Silverman,P.M. (1990) The Cpx proteins of Escherichia coli K-12: evidence that cpxA, ecfB, ssd, and eup mutations all identify the same gene *J Bacteriol.* **172**: 2456-2461.

Raivio,T.L., Laird,M.W., Joly,J.C., and Silhavy,T.J. (2000) Tethering of CpxP to the inner membrane prevents spheroplast induction of the cpx envelope stress response *Mol.Microbiol.* **37**: 1186-1197.

Raivio,T.L., Popkin,D.L., and Silhavy,T.J. (1999) The Cpx envelope stress response is controlled by amplification and feedback inhibition *J.Bacteriol.* **181**: 5263-5272.

Raivio,T.L., Silhavy,T.J. (1999) The sigmaE and Cpx regulatory pathways: overlapping but distinct envelope stress responses *Curr.Opin.Microbiol.* **2**: 159-165.

Ramirez-Santos,J., Collado-Vides,J., Garcia-Varela,M., and Gomez-Eichelmann,M.C. (2001) Conserved regulatory elements of the promoter sequence of the gene rpoH of enteric bacteria *Nucleic Acids Res.* **29**: 380-386.

Redford,P., Roesch,P.L., and Welch,R.A. (2003) DegS is necessary for virulence and is among extraintestinal Escherichia coli genes induced in murine peritonitis *Infect.Immun.* **71**: 3088-3096.

Rescigno,M., Urbano,M., Valzasina,B., Francolini,M., Rotta,G., Bonasio,R., Granucci,F., Kraehenbuhl,J.P., and Ricciardi-Castagnoli,P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria *Nat.Immunol.* **2**: 361-367.

Rezuchova,B., Kormanec,J. (2001) A two-plasmid system for identification of promoters recognized by RNA polymerase containing extracytoplasmic stress response sigma(E) in Escherichia coli *J.Microbiol.Methods* **45**: 103-111.

Rezuchova,B., Miticka,H., Homerova,D., Roberts,M., and Kormanec,J. (2003) New members of the Escherichia coli sigmaE regulon identified by a two-plasmid system *FEMS Microbiol.Lett.* **225**: 1-7.

Riesenberg-Wilmes,M.R., Bearson,B., Foster,J.W., and Curtis,R., III (1996) Role of the acid tolerance response in virulence of Salmonella typhimurium *Infect.Immun.* **64**: 1085-1092.

Riley,M. (1993) Functions of the gene products of Escherichia coli *Microbiol.Rev.* **57**: 862-952.

Rippe,K., Guthold,M., von Hippel,P.H., and Bustamante,C. (1997) Transcriptional activation via DNA-looping: visualization of intermediates in the activation pathway of E. coli RNA polymerase x sigma 54 holoenzyme by scanning force microscopy *J.Mol.Biol.* **270**: 125-138.

Rizzitello,A.E., Harper,J.R., and Silhavy,T.J. (2001) Genetic evidence for parallel pathways of chaperone activity in the periplasm of Escherichia coli *J.Bacteriol.* **183**: 6794-6800.

Robbe-Saule,V., Coynault,C., and Norel,F. (1995) The live oral typhoid vaccine Ty21a is a rpoS mutant and is susceptible to various environmental stresses *FEMS Microbiol.Lett.* **126**: 171-176.

Robbe-Saule,V., Schaeffer,F., Kowarz,L., and Norel,F. (1997) Relationships between H-NS, sigma S, SpvR and growth phase in the control of spvR, the regulatory gene of the Salmonella plasmid virulence operon *Mol.Gen.Genet.* **256**: 333-347.

Robbins,J.D., Robbins,J.B. (1984) Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of Salmonella typhi *J.Infect.Dis.* **150**: 436-449.

Robson,H.G., Vas,S.I. (1972) Resistance of inbred mice to Salmonella typhimurium *J.Infect.Dis.* **126**: 378-386.

Rollenhagen,C., Sorensen,M., Rizos,K., Hurvitz,R., and Bumann,D. (2004) Antigen selection based on expression levels during infection facilitates vaccine development for an intracellular pathogen *Proc.Natl.Acad.Sci.U.S.A* **101**: 8739-8744.

Romling,U., Bian,Z., Hammar,M., Sierralta,W.D., and Normark,S. (1998a) Curli fibers are highly conserved between Salmonella typhimurium and Escherichia coli with respect to operon structure and regulation *J.Bacteriol.* **180**: 722-731.

Romling,U., Sierralta,W.D., Eriksson,K., and Normark,S. (1998b) Multicellular and aggregative behaviour of Salmonella typhimurium strains is controlled by mutations in the agfD promoter *Mol.Microbiol.* **28**: 249-264.

Roop,R.M., Robertson,G.T., Ferguson,G.P., Milford,L.E., Winkler,M.E., and Walker,G.C. (2002) Seeking a niche: putative contributions of the hfq and bacA gene products to the successful adaptation of the brucellae to their intracellular home *Vet.Microbiol.* **%20;90**: 349-363.

Rouviere,P.E., De Las,P., Mecsas,J., Lu,C.Z., Rudd,K.E., and Gross,C.A. (1995) rpoE, the gene encoding the second heat-shock sigma factor, sigma E, in Escherichia coli *EMBO J* **14**: 1032-1042.

Rouviere,P.E., Gross,C.A. (1996) SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins *Genes Dev.* **10**: 3170-3182.

Rowe,B., Ward,L.R., and Threlfall,E.J. (1997) Multidrug-resistant Salmonella typhi: a worldwide epidemic *Clin.Infect.Dis.* **24 Suppl 1:S106-9.**: S106-S109.

Rowley,G., Stevenson,A., Kormanec,J., and Roberts,M. (2005) Effect of inactivation of degS on Salmonella enterica serovar typhimurium in vitro and in vivo *Infect.Immun.* **73**: 459-463.

Rudolph,M.G., Weise,C., Mirold,S., Hillenbrand,B., Bader,B., Wittinghofer,A., and Hardt,W.D. (1999) Biochemical analysis of SopE from Salmonella typhimurium, a highly efficient guanosine nucleotide exchange factor for RhoGTPases *J.Biol.Chem.* **274**: 30501-30509.

Ruiz-Albert,J., Yu,X.J., Beuzon,C.R., Blakey,A.N., Galyov,E.E., and Holden,D.W. (2002) Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane *Mol.Microbiol.* **44**: 645-661.

Rutherford,K., Parkhill,J., Crook,J., Horsnell,T., Rice,P., Rajandream,M.A., and Barrell,B. (2000) Artemis: sequence visualization and annotation *Bioinformatics.* **16**: 944-945.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989) *Molecular Cloning* Cold Spring Harbor Laboratory Press.

Sansonetti,P. (2001) Phagocytosis of bacterial pathogens: implications in the host response *Semin.Immunol.* **13**: 381-390.

Sansonetti,P. (2002) Host-pathogen interactions: the seduction of molecular cross talk *Gut* **50 Suppl 3**: III2-III8.

Sansonetti,P.J. (2004) War and peace at mucosal surfaces *Nat.Rev.Immunol.* **4**: 953-964.

Santos,R.L., Zhang,S., Tsolis,R.M., Kingsley,R.A., Adams,L.G., and Baumler,A.J. (2001) Animal models of Salmonella infections: enteritis versus typhoid fever *Microbes.Infect* **3**: 1335-1344.

Sasse-Dwight,S., Gralla,J.D. (1990) Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor sigma 54 *Cell* **62**: 945-954.

Schafer,U., Beck,K., and Muller,M. (1999) Skp, a molecular chaperone of gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins *J Biol Chem* **274**: 24567-24574.

Schlapschy,M., Dommel,M.K., Hadian,K., Fogarasi,M., Korndorfer,I.P., and Skerra,A. (2004) The periplasmic E. coli chaperone Skp is a trimer in solution: biophysical and preliminary crystallographic characterization *Biol.Chem.* **385**: 137-143.

Schmitt,C.K., Ikeda,J.S., Darnell,S.C., Watson,P.R., Bispham,J., Wallis,T.S., Weinstein,D.L., Metcalf,E.S., and O'Brien,A.D. (2001) Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of Salmonella enterica serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis *Infect.Immun.* **69**: 5619-5625.

Schurr,M.J., Deretic,V. (1997) Microbial pathogenesis in cystic fibrosis: co-ordinate regulation of heat-shock response and conversion to mucoidy in Pseudomonas aeruginosa *Mol.Microbiol.* **24**: 411-420.

Schurr,M.J., Martin,D.W., Mudd,M.H., and Deretic,V. (1994) Gene cluster controlling conversion to alginate-overproducing phenotype in Pseudomonas aeruginosa: functional analysis in a heterologous host and role in the instability of mucoidy *J.Bacteriol.* **176**: 3375-3382.

Schurr,M.J., Martin,D.W., Mudd,M.H., Hibler,N.S., Boucher,J.C., and Deretic,V. (1993) The algD promoter: regulation of alginate production by Pseudomonas aeruginosa in cystic fibrosis *Cell Mol.Biol.Res.* **39**: 371-376.

Schurr,M.J., Yu,H., Boucher,J.C., Hibler,N.S., and Deretic,V. (1995) Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (sigma E) which controls mucoidy in cystic fibrosis isolates of Pseudomonas aeruginosa *J Bacteriol.* **177**: 5670-5679.

Schurr,M.J., Yu,H., Martinez-Salazar,J.M., Boucher,J.C., and Deretic,V. (1996) Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and Pseudomonas aeruginosa conversion to mucoidy in cystic fibrosis *J.Bacteriol.* **178**: 4997-5004.

Schutt,C. (1999) Fighting infection: the role of lipopolysaccharide binding proteins CD14 and LBP *Pathobiology* **67**: 227-229.

Schweder,T., Lee,K.H., Lomovskaya,O., and Matin,A. (1996) Regulation of Escherichia coli starvation sigma factor (sigma s) by ClpXP protease *J.Bacteriol.* **178**: 470-476.

Selander,R.K., Beltran,P., Smith,N.H., Helmuth,R., Rubin,F.A., Kopecko,D.J., Ferris,K., Tall,B.D., Cravioto,A., and Musser,J.M. (1990) Evolutionary genetic relationships of clones of Salmonella serovars that cause human typhoid and other enteric fevers *Infect.Immun.* **58**: 2262-2275.

Shea,J.E., Hensel,M., Gleeson,C., and Holden,D.W. (1996) Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium *Proc.Natl.Acad.Sci.U.S.A* **%19;93**: 2593-2597.

Shea,J.E., Santangelo,J.D., and Feldman,R.G. (2000) Signature-tagged mutagenesis in the identification of virulence genes in pathogens *Curr.Opin.Microbiol.* **3**: 451-458.

Shin,D., Lim,S., Seok,Y.J., and Ryu,S. (2001) Heat shock RNA polymerase (E sigma(32)) is involved in the transcription of mlc and crucial for induction of the Mlc regulon by glucose in Escherichia coli *J.Biol.Chem.* **276**: 25871-25875.

Shivaprasad,H.L. (2000) Fowl typhoid and pullorum disease *Rev.Sci.Tech.* **19**: 405-424.

Shrestha,A., Shi,L., Tanase,S., Tsukamoto,M., Nishino,N., Tokita,K., and Yamamoto,T. (2004) Bacterial chaperone protein, Skp, induces leukocyte chemotaxis via C5a receptor *Am.J.Pathol.* **164**: 763-772.

Siler JF. Immunisation to Typhoid Fever. 1941. John Hopkins University Press.
Ref Type: Generic

Silva,B.A., Gonzalez,C., Mora,G.C., and Cabello,F. (1987) Genetic characteristics of the Salmonella typhi strain Ty21a vaccine *J.Infect.Dis.* **155**: 1077-1078.

Silva,J.C., Haldimann,A., Prahalad,M.K., Walsh,C.T., and Wanner,B.L. (1998) In vivo characterization of the type A and B vancomycin-resistant enterococci (VRE) VanRS two-component systems in Escherichia coli: a nonpathogenic model for studying the VRE signal transduction pathways *Proc.Natl.Acad.Sci.U.S.A* **95**: 11951-11956.

Silverman,P.M. (1982) Gene cpxA is a new addition to the linkage map of Escherichia coli K-12 *J Bacteriol.* **150**: 425-428.

Smirnova,I., Poltorak,A., Chan,E.K., McBride,C., and Beutler,B. (2000) Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4) *Genome Biol.* **1**: RESEARCH002.

Smith,G.R. (1988) Homologous recombination in procaryotes *Microbiol.Rev.* **52**: 1-28.

Smith,K.D., Andersen-Nissen,E., Hayashi,F., Strobe,K., Bergman,M.A., Barrett,S.L., Cookson,B.T., and Aderem,A. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility *Nat.Immunol.* **4**: 1247-1253.

Snoeyenbos,G.H., Smyser,C.F., and Van Roekel,H. (1969) Salmonella infections of the ovary and peritoneum of chickens *Avian Dis.* **13**: 668-670.

Snyder,W.B., Davis,L.J., Danese,P.N., Cosma,C.L., and Silhavy,T.J. (1995) Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway *J Bacteriol.* **177**: 4216-4223.

Sojka,W.J., Hudson,E.B. (1976) A survey of drug resistance in Salmonella isolated from animals in England and Wales during 1972 *Br.Vet.J.* **132**: 95-104.

Solis-Guzman,G., Ramirez-Santos,J., Souza,V., and Gomez-Eichelmann,M.C. (2001) Analysis of the regulatory region of the heat-shock gene rpoH of Escherichia coli strains isolated from non-human hosts *FEMS Microbiol.Lett.* **205**: 191-196.

Spector,M.P. (1998) The starvation-stress response (SSR) of Salmonella *Adv.Microb.Physiol.* **40**: 233-279.

Spector,M.P., Aliabadi,Z., Gonzalez,T., and Foster,J.W. (1986) Global control in Salmonella typhimurium: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins *J Bacteriol.* **168**: 420-424.

Sperber,S.J., Schleupner,C.J. (1987) Salmonellosis during infection with human immunodeficiency virus *Rev.Infect.Dis.* **9**: 925-934.

Spiess,C., Beil,A., and Ehrmann,M. (1999) A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein *Cell* **97**: 339-347.

Spinola,S.M., Hiltke,T.J., Fortney,K., and Shanks,K.L. (1996) The conserved 18,000-molecular-weight outer membrane protein of Haemophilus ducreyi has homology to PAL *Infect.Immun.* **64**: 1950-1955.

Staats,H.F., Jackson,R.J., Marinaro,M., Takahashi,I., Kiyono,H., and McGhee,J.R. (1994) Mucosal immunity to infection with implications for vaccine development *Curr.Opin.Immunol.* **6**: 572-583.

Steele-Mortimer,O., Meresse,S., Gorvel,J.P., Toh,B.H., and Finlay,B.B. (1999) Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway *Cell Microbiol.* **1**: 33-49.

Stender,S., Friebel,A., Linder,S., Rohde,M., Mirold,S., and Hardt,W.D. (2000) Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell *Mol.Microbiol.* **36**: 1206-1221.

Stock,A., Chen,T., Welsh,D., and Stock,J. (1988) CheA protein, a central regulator of bacterial chemotaxis, belongs to a family of proteins that control gene expression in response to changing environmental conditions *Proc.Natl.Acad.Sci.U.S.A* **85**: 1403-1407.

Stock,A.M., Robinson,V.L., and Goudreau,P.N. (2000) Two-component signal transduction *Annu.Rev.Biochem.* **69:183-215.**: 183-215.

Stocker,B.A. (1990) Aromatic-dependent Salmonella as live vaccine presenters of foreign epitopes as inserts in flagellin *Res.Microbiol.* **141**: 787-796.

Stoleru,G.H., Le Minor,L., and Lheritier,A.M. (1976) Polynucleotide sequence divergence among strains of Salmonella sub-genus IV and closely related organisms *Ann.Microbiol.(Paris)* **127**: 477-486.

Strauch,K.L., Johnson,K., and Beckwith,J. (1989) Characterization of degP, a gene required for proteolysis in the cell envelope and essential for growth of Escherichia coli at high temperature *J.Bacteriol.* **171**: 2689-2696.

Su,W., Porter,S., Kustu,S., and Echols,H. (1990) DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter *Proc.Natl.Acad.Sci.U.S.A* **87**: 5504-5508.

Sutton,A., Newman,T., McEwen,J., Silverman,P.M., and Freundlich,M. (1982) Mutations in genes cpxA and cpxB of Escherichia coli K-12 cause a defect in acetohydroxyacid synthase I function in vivo *J Bacteriol.* **151**: 976-982.

Sydenham,M., Douce,G., Bowe,F., Ahmed,S., Chatfield,S., and Dougan,G. (2000) Salmonella enterica serovar typhimurium surA mutants are attenuated and effective live oral vaccines *Infect.Immun.* **68**: 1109-1115.

Szu,S.C., Stone,A.L., Robbins,J.D., Schneerson,R., and Robbins,J.B. (1987) Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in laboratory animals *J Exp.Med.* **166**: 1510-1524.

Tacket,C.O., Hone,D.M., Curtiss,R., III, Kelly,S.M., Losonsky,G., Guers,L., Harris,A.M., Edelman,R., and Levine,M.M. (1992a) Comparison of the safety and immunogenicity of delta aroC delta aroD and delta cya delta crp Salmonella typhi strains in adult volunteers *Infect.Immun.* **60**: 536-541.

Tacket,C.O., Hone,D.M., Losonsky,G.A., Guers,L., Edelman,R., and Levine,M.M. (1992b) Clinical acceptability and immunogenicity of CVD 908 Salmonella typhi vaccine strain *Vaccine* **10**: 443-446.

Tanaka,K., Takayanagi,Y., Fujita,N., Ishihama,A., and Takahashi,H. (1993) Heterogeneity of the principal sigma factor in Escherichia coli: the rpoS gene product, sigma 38, is a second principal sigma factor of RNA polymerase in stationary-phase Escherichia coli *Proc.Natl.Acad.Sci.U.S.A* **90**: 3511-3515.

Tapa,S., Cvjetanovic,B. (1975) Controlled field trial on the effectiveness of one and two doses of acetone-inactivated and dried typhoid vaccine *Bull.World Health Organ* **52**: 75-80.

Tatsuta,T., Joob,D.M., Calendar,R., Akiyama,Y., and Ogura,T. (2000) Evidence for an active role of the DnaK chaperone system in the degradation of sigma(32) *FEBS Lett.* **478**: 271-275.

Tatsuta,T., Tomoyasu,T., Bukau,B., Kitagawa,M., Mori,H., Karata,K., and Ogura,T. (1998) Heat shock regulation in the ftsH null mutant of Escherichia coli: dissection of stability and activity control mechanisms of sigma32 in vivo *Mol.Microbiol.* **30**: 583-593.

Testerman,T.L., Vazquez-Torres,A., Xu,Y., Jones-Carson,J., Libby,S.J., and Fang,F.C. (2002) The alternative sigma factor sigmaE controls antioxidant defences required for Salmonella virulence and stationary-phase survival *Mol.Microbiol.* **43**: 771-782.

Thatte,J., Rath,S., and Bal,V. (1993) Immunization with live versus killed Salmonella typhimurium leads to the generation of an IFN-gamma-dominant versus an IL-4-dominant immune response *Int.Immunol.* **5**: 1431-1436.

Thome,B.M., Muller,M. (1991) Skp is a periplasmic Escherichia coli protein requiring SecA and SecY for export *Mol.Microbiol.* **5**: 2815-2821.

Threlfall,E.J. (2002) Antimicrobial drug resistance in Salmonella: problems and perspectives in food- and water-borne infections *FEMS Microbiol.Rev.* **26**: 141-148.

Threlfall,E.J., Frost,J.A., Ward,L.R., and Rowe,B. (1996) Increasing spectrum of resistance in multiresistant Salmonella typhimurium *Lancet* **347**: 1053-1054.

Threlfall,E.J., Graham,A., Cheasty,T., Ward,L.R., and Rowe,B. (1997a) Resistance to ciprofloxacin in pathogenic Enterobacteriaceae in England and Wales in 1996 *J.Clin.Pathol.* **50**: 1027-1028.

Threlfall,E.J., Ward,L.R., and Rowe,B. (1997) Increasing incidence of resistance to trimethoprim and ciprofloxacin in epidemic Salmonella typhimurium DT104 in England and Wales *Euro.Surveill* **2**: 81-84.

Threlfall,E.J., Ward,L.R., Skinner,J.A., and Rowe,B. (1997b) Increase in multiple antibiotic resistance in nontyphoidal salmonellas from humans in England and Wales: a comparison of data for 1994 and 1996 *Microb.Drug Resist.* **3**: 263-266.

Tilly,K., Erickson,J., Sharma,S., and Georgopoulos,C. (1986) Heat shock regulatory gene rpoH mRNA level increases after heat shock in Escherichia coli *J.Bacteriol.* **168**: 1155-1158.

Tomoyasu,T., Takaya,A., Sasaki,T., Nagase,T., Kikuno,R., Morioka,M., and Yamamoto,T. (2003) A new heat shock gene, AgsA, which encodes a small chaperone involved in suppressing protein aggregation in Salmonella enterica serovar typhimurium *J.Bacteriol.* **185**: 6331-6339.

Tormo,A., Almiron,M., and Kolter,R. (1990) surA, an Escherichia coli gene essential for survival in stationary phase *J.Bacteriol.* **172**: 4339-4347.

Uchiya,K., Barbieri,M.A., Funato,K., Shah,A.H., Stahl,P.D., and Groisman,E.A. (1999) A Salmonella virulence protein that inhibits cellular trafficking *EMBO J.* **18**: 3924-3933.

Vazquez-Torres,A., Jones-Carson,J., Baumler,A.J., Falkow,S., Valdivia,R., Brown,W., Le,M., Berggren,R., Parks,W.T., and Fang,F.C. (1999) Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes *Nature* **401**: 804-808.

Vianney,A., Lewin,T.M., Beyer,W.F., Jr., Lazzaroni,J.C., Portalier,R., and Webster,R.E. (1994) Membrane topology and mutational analysis of the TolQ protein of Escherichia coli required for the uptake of macromolecules and cell envelope integrity *J.Bacteriol.* **176**: 822-829.

Vianney,A., Muller,M.M., Clavel,T., Lazzaroni,J.C., Portalier,R., and Webster,R.E. (1996) Characterization of the tol-pal region of Escherichia coli K-12: translational control of tolR expression by TolQ and identification of a new open reading frame downstream of pal encoding a periplasmic protein *J.Bacteriol.* **178**: 4031-4038.

Villarreal-Ramos,B., Manser,J.M., Collins,R.A., Chance,V., Eckersall,D., Jones,P.W., and Dougan,G. (2000) Susceptibility of calves to challenge with Salmonella typhimurium 4/74 and derivatives harbouring mutations in htrA or purE *Microbiology* **146**: 2775-2783.

Vincent,P.A., Delgado,M.A., Farias,R.N., and Salomon,R.A. (2004) Inhibition of Salmonella enterica serovars by microcin J25 *FEMS Microbiol.Lett.* **236**: 103-107.

Wall,P.G., Morgan,D., Lamden,K., Ryan,M., Griffin,M., Threlfall,E.J., Ward,L.R., and Rowe,B. (1994) A case control study of infection with an epidemic strain of multiresistant Salmonella typhimurium DT104 in England and Wales *Commun.Dis.Rep.CDR Rev.* **4**: R130-R135.

Waller,P.R., Sauer,R.T. (1996) Characterization of degQ and degS, Escherichia coli genes encoding homologs of the DegP protease *J.Bacteriol.* **178**: 1146-1153.

Wallis,T.S., Galyov,E.E. (2000) Molecular basis of Salmonella-induced enteritis *Mol.Microbiol.* **36**: 997-1005.

Walsh,N.P., Alba,B.M., Bose,B., Gross,C.A., and Sauer,R.T. (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain *Cell* **113**: 61-71.

Walton,T.A., Sousa,M.C. (2004) Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation *Mol.Cell* **15**: 367-374.

Wang,Q.P., Kaguni,J.M. (1989) A novel sigma factor is involved in expression of the rpoH gene of Escherichia coli *J.Bacteriol.* **171**: 4248-4253.

Wang,R.F., Kushner,S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli *Gene* **100:195-9**: 195-199.

Wanner,B.L. (1992) Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? *J.Bacteriol.* **174**: 2053-2058.

Wanner,B.L., Latterell,P. (1980) Mutants affected in alkaline phosphatase, expression: evidence for multiple positive regulators of the phosphate regulon in Escherichia coli *Genetics* **96**: 353-366.

Warner,J.B., Lolkema,J.S. (2002) LacZ-promoter fusions: the effect of growth *Microbiology* **148**: 1241-1243.

Waterman,S.R., Holden,D.W. (2003) Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system *Cell Microbiol.* **5**: 501-511.

Weber,R.F., Silverman,P.M. (1988) The cpx proteins of Escherichia coli K12. Structure of the cpxA polypeptide as an inner membrane component *J Mol.Biol.* **203**: 467-478.

Webster,R.E. (1991) The tol gene products and the import of macromolecules into Escherichia coli *Mol.Microbiol.* **5**: 1005-1011.

Weiner,L., Brissette,J.L., and Model,P. (1991) Stress-induced expression of the Escherichia coli phage shock protein operon is dependent on sigma 54 and modulated by positive and negative feedback mechanisms *Genes Dev.* **5**: 1912-1923.

Weiner,L., Brissette,J.L., Ramani,N., and Model,P. (1995) Analysis of the proteins and cis-acting elements regulating the stress-induced phage shock protein operon *Nucleic Acids Res.* **23**: 2030-2036.

Weiner,L., Model,P. (1994) Role of an Escherichia coli stress-response operon in stationary-phase survival *Proc.Natl.Acad.Sci.U.S.A* **91**: 2191-2195.

Weiss,D.S., Batut,J., Klose,K.E., Keener,J., and Kustu,S. (1991) The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription *Cell* **67**: 155-167.

West,A.H., Stock,A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems *Trends Biochem.Sci.* **26**: 369-376.

Whitchurch,C.B., Erova,T.E., Emery,J.A., Sargent,J.L., Harris,J.M., Semmler,A.B., Young,M.D., Mattick,J.S., and Wozniak,D.J. (2002) Phosphorylation of the

Pseudomonas aeruginosa response regulator AlgR is essential for type IV fimbria-mediated twitching motility *J.Bacteriol.* **184**: 4544-4554.

Williams,C.H., Jr. (1995) Mechanism and structure of thioredoxin reductase from Escherichia coli *FASEB J.* **9**: 1267-1276.

Williams,C.H., Arscott,L.D., Muller,S., Lennon,B.W., Ludwig,M.L., Wang,P.F., Veine,D.M., Becker,K., and Schirmer,R.H. (2000) Thioredoxin reductase two modes of catalysis have evolved *Eur.J.Biochem.* **267**: 6110-6117.

Wilson,R.B., Davis,D., and Mitchell,A.P. (1999) Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions *J.Bacteriol.* **181**: 1868-1874.

Winfield,M.D., Groisman,E.A. (2003) Role of nonhost environments in the lifestyles of Salmonella and Escherichia coli *Appl.Environ.Microbiol.* **69**: 3687-3694.

Winfield,M.D., Groisman,E.A. (2004) Phenotypic differences between Salmonella and Escherichia coli resulting from the disparate regulation of homologous genes *Proc.Natl.Acad.Sci.U.S.A* **101**: 17162-17167.

Wong,C., Tintut,Y., and Gralla,J.D. (1994) The domain structure of sigma 54 as determined by analysis of a set of deletion mutants *J.Mol.Biol.* **236**: 81-90.

Wong,K.K., McClelland,M., Stillwell,L.C., Sisk,E.C., Thurston,S.J., and Saffer,J.D. (1998) Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a Salmonella pathogenicity island located at 92 minutes on the chromosome map of Salmonella enterica serovar typhimurium LT2 *Infect.Immun.* **66**: 3365-3371.

Wood,M.W., Jones,M.A., Watson,P.R., Hedges,S., Wallis,T.S., and Galyov,E.E. (1998) Identification of a pathogenicity island required for Salmonella enteropathogenicity *Mol.Microbiol.* **29**: 883-891.

Wozniak,D.J., Ohman,D.E. (1994) Transcriptional analysis of the Pseudomonas aeruginosa genes algR, algB, and algD reveals a hierarchy of alginate gene expression which is modulated by algT *J.Bacteriol.* **176**: 6007-6014.

Wray,C., Sojka,W.J. (1978) Experimental Salmonella typhimurium infection in calves *Res.Vet.Sci.* **25**: 139-143.

Wright,S.D., Ramos,R.A., Tobias,P.S., Ulevitch,R.J., and Mathison,J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein *Science* **249**: 1431-1433.

Wyant,T.L., Tanner,M.K., and Sztein,M.B. (1999) Salmonella typhi flagella are potent inducers of proinflammatory cytokine secretion by human monocytes *Infect.Immun.* **67**: 3619-3624.

Wyckoff,T.J., Raetz,C.R., and Jackman,J.E. (1998) Antibacterial and anti-inflammatory agents that target endotoxin *Trends Microbiol.* **6**: 154-159.

Xie,Z.D., Hershberger,C.D., Shankar,S., Ye,R.W., and Chakrabarty,A.M. (1996) Sigma factor-anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA *J Bacteriol.* **178**: 4990-4996.

Xu,H.R., Hsu,H.S., Moncure,C.W., and King,R.A. (1993a) Correlation of antibody titres induced by vaccination with protection in mouse typhoid *Vaccine* **11**: 725-729.

Xu,H.R., Tan,Y.Y., Hsu,H.S., Moncure,C.W., and Wang,X.M. (1993b) Comparative antibody response to Salmonella antigens in genetically resistant and susceptible mice *Clin.Exp.Immunol.* **91**: 73-77.

Xu,J., Johnson,R.C. (1995a) Fis activates the RpoS-dependent stationary-phase expression of proP in Escherichia coli *J.Bacteriol.* **177**: 5222-5231.

Xu,J., Johnson,R.C. (1995b) Identification of genes negatively regulated by Fis: Fis and RpoS comodulate growth-phase-dependent gene expression in Escherichia coli *J.Bacteriol.* **177**: 938-947.

Yaku,H., Mizuno,T. (1997) The membrane-located osmosensory kinase, EnvZ, that contains a leucine zipper-like motif functions as a dimer in Escherichia coli *FEBS Lett.* **417**: 409-413.

Yamamoto,K., Hirao,K., Oshima,T., Aiba,H., Utsumi,R., and Ishihama,A. (2004) Functional characterization in vitro of all two-component signal transduction systems from Escherichia coli *J.Biol.Chem.* .

Yeats,C., Bateman,A. (2003) The BON domain: a putative membrane-binding domain *Trends Biochem.Sci.* **28**: 352-355.

Yorgey,P., Kolter,R. (1993) A widely conserved developmental sensor in bacteria? *Trends Genet.* **9**: 374-375.

Yu,H., Boucher,J.C., Hibler,N.S., and Deretic,V. (1996) Virulence properties of Pseudomonas aeruginosa lacking the extreme-stress sigma factor AlgU (sigmaE) *Infect Immun* **64**: 2774-2781.

Yu,H., Schurr,M.J., and Deretic,V. (1995) Functional equivalence of Escherichia coli sigma E and Pseudomonas aeruginosa AlgU: E. coli rpoE restores mucoidy and reduces sensitivity to reactive oxygen intermediates in algU mutants of P. aeruginosa *J Bacteriol.* **177**: 3259-3268.

Yu,J. (1998) Inactivation of DsbA, but not DsbC and DsbD, affects the intracellular survival and virulence of Shigella flexneri *Infect Immun.* **66**: 3909-3917.

Yu,J., Kroll,J.S. (1999) DsbA: a protein-folding catalyst contributing to bacterial virulence *Microbes.Infect.* **1**: 1221-1228.

Yu,J., Webb,H., and Hirst,T.R. (1992) A homologue of the Escherichia coli DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in Vibrio cholerae *Mol.Microbiol* **6**: 1949-1958.

Zhang,S., Santos,R.L., Tsolis,R.M., Stender,S., Hardt,W.D., Baumler,A.J., and Adams,L.G. (2002) The Salmonella enterica serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves *Infect.Immun.* **70**: 3843-3855.

Zhang,Y., Buchholz,F., Muyrers,J.P., and Stewart,A.F. (1998) A new logic for DNA engineering using recombination in Escherichia coli *Nat.Genet.* **20**: 123-128.

Zhou,D., Mooseker,M.S., and Galan,J.E. (1999) Role of the S. typhimurium actin-binding protein SipA in bacterial internalization *Science* **283**: 2092-2095.

Zhou,Y.N., Kusukawa,N., Erickson,J.W., Gross,C.A., and Yura,T. (1988) Isolation and characterization of Escherichia coli mutants that lack the heat shock sigma factor sigma 32 *J.Bacteriol.* **170**: 3640-3649.

APPENDIX

The appendix consists of the entire microarray dataset located on the CD attached to the back cover.